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STUDIES OF EFFECTS OF PARTICLE SIZE ON THE TOXICITY OF INSECTICIDE AEROSOLS

FINAL REPORT

Peter E. Berteau,

Wallace A. Deen and Robert L. Dimmick

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Inhalation toxicity values for aerosols of four insecticides (chlorpyrifos, malathion, naled and resmethrin) have been expressed as LD50 values in mg/kg body-weight. To calculate this value we used the aerosol concentration (mg/l), time of exposure (min), respiratory minute volume of the animal (ml/min/g) and whole-body retention of inhaled material as estimated from date generated when an aerosol containing a tracer was inhaled. The inhalation LD50

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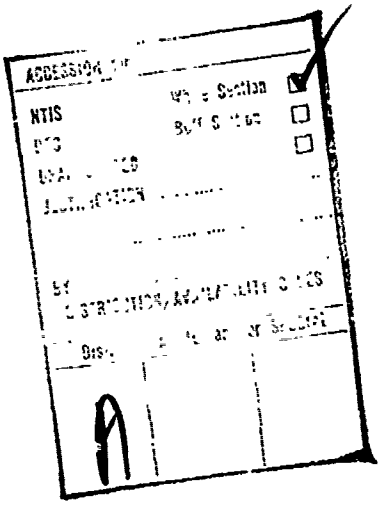
was compared with the oral LD50 for four pesticide aerosols of mass median diameter 2.1 µm. By this method of comparison chlorpyrifos was slightly more toxic to rats by the inhalation than by the oral route but naled was about 21 times more toxic by the inhalation route. Less marked differences between the two routes of administration were observed with mice. When the aerosol particle size was increased to mass median diameter 18 - 20 µm, the toxicity of naled was reduced by at least one-fourth. Malathion and resmethrin aerosols were insufficiently toxic to kill animals by practicable acute inhalation exposure. Significant plasma cholinesterase depression was observed when animals were exposed to sub-lethal doses of either chlorpyrifos or naled aerosols.



Studies of Effects of Particle Size on the Toxicity of Insecticide Aerosols

Summary and Principal Accomplishments

The purpose of this project was to determine whether the inhalation toxicity of four selected pesticides was greater for animals than the oral toxicity, and whether particle size played an important role in the toxicity. Pesticides studied were chlorpyrifos, malathion, naled and resmethrin.

To accomplish these purposes, we did the following:

1. Modified a Henderson chamber to allow effective exposure of animals to small-particle aerosols of pesticides in the 0.8 μm to 5 μm range.

2. Conducted 193 aerosol exposures of either rats or mice.

3. Measured the retention of aerosols in rats and mice of the particle size being used.

4. Determined acute inhalation LCt50 values, calculated inhalation LD50 values in mg/kg body weight for two pesticides with small-particle aerosols, and found the two other pesticides were not toxic enough to establish inhalation LD50's under feasible exposure conditions. To determine these values, use was made of the aerosol concentration in air, time of exposure, respiratory minute volume of the animal and retention and early distribution of a tracer aerosol of comparable particle size.

5. Devised and built an exposure chamber, and a dispenser to allow tests to be conducted with large-particle aerosols (13 μm - 20 μm diameter). Ancillary to these tests, perfected a method for measuring this size range, and produced inexpensive holding units for head-only exposure of rats.

6. Conducted tests with the above-mentioned aerosol to show that large particles (18 μm - 20 μm) were less toxic than small particles in the case of one pesticide.

7. Provided supplementary data to show that there is probably a concentration-per-particle effect that can influence calculated inhalation LD50 values; and that particles in the 2 μm - 5 μm range may be about as toxic as particles less than 2 μm.

8. Conducted tests on exposed animals to correlate dosages with extent of changes of certain biochemical parameters, such as plasma cholinesterase depression and changes in whole blood serotonin or glutathione.

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FOREWORD

The U.S. Army is considering the use of ultra-low volume (ULV) sprayers in the place of conventional foggers for several pesticide application programs. The impact which smaller, more concentrated pesticide aerosols have on non-target organisms (including man) must be considered for each potential application. This study was initiated to provide information from which the application of ULV techniques for specific pesticides could be determined. The primary objective was to determine the relative toxicological properties of four insecticides when test animals*were exposed to "respirable" and "non-respirable" aerosols. Secondary objectives were to compare the toxicological properties of these pesticides after animals had been exposed by different routes (principally oral and inhalation) and to attempt to define the mechanisms that might have caused differences in the resulting data.

Assessment of toxicological hazards to non-target organisms when pesticides are applied in the field has largely been based upon reported information on the LD50 values for these pesticides. These values are available for almost all pesticide chemicals based upon the toxicity when animals received oral doses. Such values may have been used by regulatory agencies to assess the hazard to field workers. However, the exposure of such persons must largely be by the inhalation route; thus, there is need to know the true inhalation dose that such an individual might receive. Inhalation toxicity is conventionally expressed as a Cxt value or LC50 x t, i.e., the lethal concentration (c) of the chemical in the air times the time (t) of exposure. Such a term is largely empirical and is of little use to enable valid comparisons to be made with reported values of oral, intraperitoneal, intravenous, etc., toxicity where the animals can be assumed to have received all the amount of chemical administered.

In this report, therefore, we have expanded the term "LD50" to include measures of inhalation toxicity expressed as mg/kg body-weight. Such an expression is in keeping with the terminology encountered in some recent toxicological reports (e.g., McFarland, 1975; Hoben, et al., 1976). To enable such an expression to be made, in addition to the Ct value, we must also know the respiratory minute volume of the animal, preferably under the conditions of exposure and also the retention and early distribution of the inhaled air or aerosol in the whole body.

For respiratory minute volumes we have used the values reported by Guyton (1947) which were allegedly measured under resting conditions. However, in order to perform such measurements, the animal must be placed in a plethysmograph which, by its very restrictive nature, induces considerable stress. It is therefore our opinion that the values reported by Guyton are accurate enough to be applicable to our conditions of exposure.

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*In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

For retention values (of the total inhaled) we have used our own experimental data based upon the levels of an inert radiolabeled tracer found in body organs of animals after they have inhaled measured levels of the aerosol. The tracer was formulated in vegetable oil, used as a vehicle for many of our exposures.

MATERIALS AND METHODS

For these studies four insecticides of current importance to the U.S. Army for field studies were examined in laboratory animals. These chemicals were the three organophosphorus insecticides, chlorpyrifos (Dursban®), malathion and naled (Dibrom®), and the pyrethroid resmethrin*. Details of the formulations are given in Table 1. Chemical structures are shown in Figure 1.

1. Animal exposure techniques

All exposures reported were performed with female mice (NAMRU strain, 20-30 g weight) or female Sprague Dawley rats (250-350 g).

1.1 Inhalation exposure

1.1.1. Small particles (0.8-5 μm) 3 μm MMD

Small particle aerosols were generated from formulations of pesticide chemicals dispersed from two refluxing Wells atomizers operated in parallel. The aerosols were conducted into a rectangular chamber (Henderson, 1952) containing 8-16 female mice or 8 female rats. Doses were varied by varying the exposure time. Details of the exposure methods and calculation of the dose are given in Appendix 1.

1.1.2. Medium sized particles (ca. 8.0 μm MMD)

Attempts to generate aerosols of this particle size range were without success. Further details of these attempts are given in Appendix 2.

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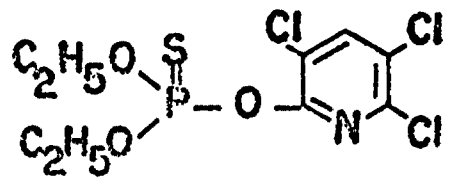
* When referring to the insecticide chemical, generic names will henceforth be used throughout the text. Certain formulations may be identified by the use of trade names (e.g., Dibrom 14 concentrate). Use of such names does not imply endorsement of the products by either the sponsoring agencies or by the University of California.

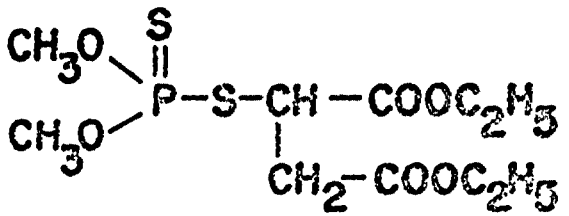
Table 1. Commercial formulations of insecticides used in this study

Pesticide chemical	Formulation adjuvant	Trade Name	Concentration of active ingredient[a]
chlorpyrifos	xylene	Dow Mosquito Fogging Concentrate (Dursban®)	65% w/w
malathion	"inert ingredients"	malathion	95% technical grade
naled	aromatic hydrocarbons	Dibrom 14 concentrate	87% w/w
resmethrin	Panasol AN 2	SBP - 1382	40% w/w

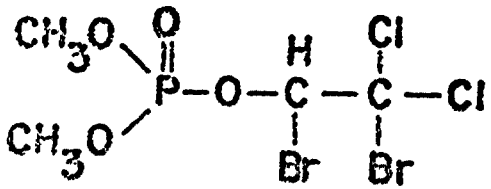
[a] Concentrations vary slightly with each batch formulated.



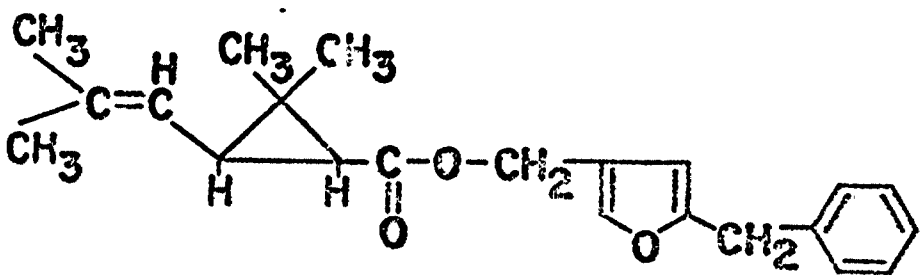
chlorpyrifos



malathion



naled



resmethrin

Figure 1. Chemical structures of insecticides under study.

1.1.3 Large particles (18 - 20 μm MMD)

A modification of the May (1949) air-driven spinning top was used to generate aerosols in this particular size range. However, to obtain sufficient aerosol concentration, a larger, electrically-driven spinning disk was used. The particles were allowed to descend under a lightly generated air flow to the animals (8 rats) placed in head-only exposure holders. Details of the generation of these aerosols and method of exposure are also given in Appendix 2.

1.2 Oral exposure

The procedure was essentially the same as that described by Gaines (1968). Mice were sized so that groups of 10 or 16 did not vary by more than 1 g in weight. In the case of rats a weight variation of 10 g or less was acceptable for animals of 250 g or over. Solutions of the pesticide were prepared in vegetable oil (soya-bean oil or peanut oil) such that 0.5 ml or 0.1 ml contained the desired dosage. The solution was administered by oral intubation using a blunt-edged syringe needle. Mortality was observed over a 14-day post-exposure period.

1.3 Intraperitoneal exposure

In order for a valid comparison of other routes of administration with the inhalation route to be made, an intraperitoneal LD50 was obtained by injecting various doses of Dibrom 14 concentrate diluted in 0.5 ml of 1,2-propylene glycol into the peritoneal cavity. Five dose levels were used, the maximum concentration being 2.75 w/v. Mortality was normally rapid after we injected the animals; however, surviving animals were held for observation for 14 days.

2. Parameters of toxicity

2.1 Mortality and visible signs

After animals had been exposed for selected times to measured aerosol masses, the number of dead animals was noted and live animals were housed for 14 days to determine delayed mortality. A similar follow-up was made when oral and intraperitoneal routes were tested. The LD50 values and 95% confidence intervals for all routes of administration were determined by plotting the percentage mortality against dose and using the statistical

method of Litchfield and Wilcoxon (1949).

In the case of the organophosphorus insecticides, symptoms signifying cholinergic effects were noted.

2.2 Biochemical parameters

2.2.1 Cholinesterase determinations

Plasma cholinesterase determinations were made on mice or rats exposed to organophosphorus insecticides. Blood was obtained from the eye (mouse) or tail (mouse or rat) for determination before and after exposure to the insecticide aerosols. The method used was essentially that of Wolfsie and Winter (1952), which is a microadaptation of the potentiometric method of Michel (1949). Details of the method of determination are given in Appendix 3. The ratio of ΔpH/hr values after and before exposure, multiplied by 100, gives a figure which is indicative of cholinesterase depression, and is expressed as cholinesterase percentage of pre-exposure value. A figure of 80% or less was considered significant evidence of cholinergic activity.

2.2.2 Serotonin determinations

Serotonin elevation in whole blood has been reported to be elevated in animals exposed to certain pesticides (e.g., Shilina, 1973). The method of determining the level in whole blood of rats or mice, before and after inhalation exposure to the insecticide aerosol, is essentially the modification by Krueger et al. (1963) of the method of Udenfriend et al. (1955). Details are given in Appendix 4.

2.2.3 Glutathione determinations

Attempts were made to measure the effect of insecticide aerosol exposure on whole blood glutathione in mice or rats. Details are given in Appendix 5.

3. Particle Size Determinations

With the small particle aerosols (3 μm MMD) the six stage Andersen sampler, operated at 1 ft^3/min and backed by a 0.8 μm Millipore* filter after the final stage, proved useful for obtaining the particle size distribution. For larger particles the sampler was modified and calibrated for use at a lower flow rate. In Appendix 6 we have discussed the use of the sampler for various particle size aerosols.

* Millipore Corporation, Bedford, Massachusetts

4. Determination of whole-body retention of aerosols

As explained in the Introduction section, in order to determine the true inhalation dose of a chemical (and hence calculate an inhalation LD50 value in mg/kg body-weight) it is necessary to know, among other terms, the percentage of inhaled aerosol which is retained in various organs of the animal. To make these measurements, mice or rats were exposed in a rectangular Henderson chamber to a typical formulation vehicle (soya-bean oil) containing a small amount of a relatively inert radio-labeled tracer (1-^{14}C-heptadecane). Details of the exposure method and calculation of the retention in the organs and tissues examined are given in Appendix 6.

5. Miscellaneous determinations

Because of the varied nature of the experimental work described above, a number of observations were made which encouraged us to perform several ancillary experiments, some of which provide a peripheral, although interesting, corollary to this study. Among these were (a) modifying methods of analysis sometimes used to determine the purity or the concentration of pesticides in aerosols; (b) the effect of a radioactive source (krypton^{85}) on the dispersion of an electrical charge on an aerosol; (c) the effect of xylene on the inhalation toxicity of chlorpyrifos, and (d) the effect of "stripping" of the particles above a certain size in the small particle aerosols. Details of the experimental procedures involved in these studies are given in Appendix 7.

RESULTS

1. Retention of small particle aerosols in animals

Table 2 summarizes the retention levels of small particle aerosols of soya-bean oil in various organs in mice and rats. Although the majority (84.1%) of the calculated inhaled material was retained in the mouse, only 28.3% was recovered in the rat. The significance of this difference between the two species is discussed below.

2. Toxicity data

2.1 Mortality

2.1.1 Comparison of oral and inhalation administration

In Table 3 , the general conditions of exposure to animals in the Henderson chamber are given. For acute exposure, times in excess of two hours were generally avoided, except when there was no indication of mortality, as in the case of malathion. In all these exposures in the Henderson chamber the aerosol concentrations were quite high (4-8 mg/l) but below those at which appreciable particle coagulation might be expected to occur.

Table 4 lists the oral and inhalation toxicity values obtained from all insecticides when animals were exposed to given formulations of the insecticides of MMD about 2 μm. The obvious increase in toxicity of naled to rats when administered by the inhalation route as contrasted to the oral route is discussed below. Because we were unable to kill mice with malathion or resmethrin aerosols, we did not attempt to expose rats to these pesticides.

There was evidence that the adjuvant used in the standard formulation (40% resmethrin in Panasol -- an alkyl naphthalene compound) was more toxic than the insecticidal ingredient. Nine of 10 mice exposed for five hours to an aerosol of 40% material died (total aerosol concentration 5.92 mg/l) whereas 16 out of 16 mice died when exposed for five hours to an aerosol of pure Panasol (aerosol concentration 6.13 mg/l). Attempts were made to formulate resmethrin in solvents other than Panasol (xylene, 1,2-propylene glycol or soya-bean oil) but the compounds were either insufficiently soluble or the solvent was too toxic to be useful.

Table 2.

Mean percentage, with 95% confidence intervals, of retention in various tissues of total calculated inhaled aerosol mass in mice and rats[a]

	Exposure method		
	Mouse[b,c]		Rat[b,c]
Tissue	Whole body	Head only	Whole body
Head	9.1 ± 3.4	-	3.1 ± 0.1
Lung	3.8 ± 0.46	4.6 ± 0.86	9.9 ± 2.7
Trachea	0.87± 0.91	1.2 ± 0.91	0.16± 0.07
Stomach	59 ± 30	40 ± 13	13 ± 7.7
Esophagus	4.7 ± 9.7	1.4 ± 0.75	0.39± 0.47
Duodenum	7.0 ± 5.8	-	1.9 ± 1.3
Total	84 ± 22	47 ± 13[d]	28 ± 9.3

[a]Mass median diameter, 2.1 μm.

[b]Females only.

[c]Eight animals per group exposed.

[d]Concentrations in head and duodenum were not determined.

TABLE 3.

CONDITIONS OF INHALATION EXPOSURE OF MICE AND RATS

TO AEROSOLS[a] OF FOUR INSECTICIDE FORMULATIONS

Species	Pesticide Chemical	Formulation	Number of exposure to determine LD_{50}	No. of animals per exposure	Duration of exposure (range)(min)	Aerosol Concentration (range) (mg/l)
Mouse	chlorpyrifos	65% in xylene	6	16	27 - 50	6.6 - 7.9
Mouse	malathion	95% technical grade	1	16	300	6.9
Mouse	naled	10 or 20% Dibrom 14 conc. in soya-bean oil	8	10	72 - 111	5.6 - 6.1
Mouse	resmethrin	40% in xylene	2	16	96 - 177	6.5 - 6.8
Rat	chlorpyrifos	65% in xylene	5	8	60 - 180	5.9 - 7.5
Rat	naled	10% Dibrom 14 conc. in soya-bean oil	5	8	48 - 61	4.7 - 5.5

[a] Small particle aerosols only (MMD $\simeq$ 2 µm) - see Table 5.

Figures 2-5 are typical dose-response data resulting from the administration of these insecticide formulations to mice or rats by either the oral or inhalation route.

For our own information, a limited number of tissue samples of animals exposed to naled were collected to assess the possibility of correlating toxicity with pathological lesions. Primary lesions in exposed animals were those of pulmonary congestion and serous edema. Pulmonary lesions were most severe in animals exposed to naled dissolved in organic solvents, but were not readily distinguishable from those lungs of animals exposed to the organic solvent alone.

Congestion and edema appeared less severe in animals exposed to naled dissolved in soya-bean oil but appeared to increase in severity with the increase in concentration of naled in the soya-bean oil. Further experiments would be required to verify fully this relationship.

2.1.2 Effect of particle size and concentration on toxicity

The inhalation LD50 for rats exposed to small particle aerosols (MMD 2.1 µm) of naled, formulated as 10% $^{w}/_{v}$ of Dibrom 14 concentrate in soya-bean oil, was 7.7 mg/kg (7.2 - 8.4) (Table 4). The inhalation LD50 for rats exposed to small particle aerosols (MMD 2.1 µm) of undiluted Dibrom 14 concentrate was 3.1 mg/kg (2.5 - 4.0). The purpose of obtaining the latter value was to compare, on a more equal basis, changes in the LD50 value for particles 13 - 20 µm in diameter, where we had to use the Dibrom 14 concentrate to obtain any death of exposed rats. That value was >12.4 mg/kg (25% mortality at this level; Table 5). Thus, large particles of the concentrate must be about half as toxic (mg/kg basis) as small particles of the 10% formulation, but about 4 times less toxic than small particles of the concentrate. One attempt was made to generate a large particle aerosol with 65% chlorpyrifos in xylene (Dow Mosquito Fogging Agent); no rats died following 147 min exposure to a concentration of 0.45 mg/l (estimated dose 12.2 mg/kg); however these particles were smaller (MMD 8.0 µm) than aerosols from naled.

Dose response curves relating to oral administration of four insecticides to female mice.

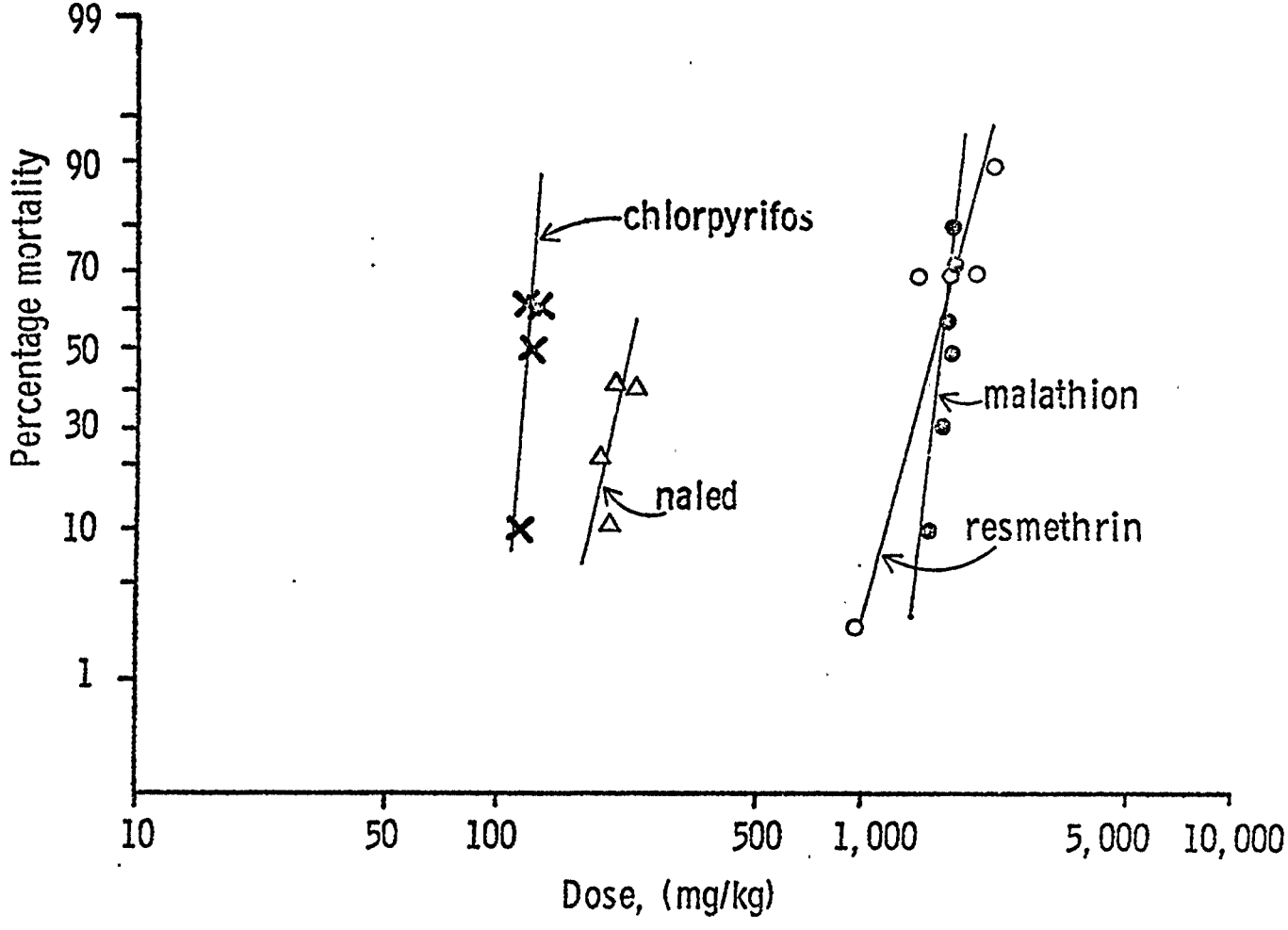


Figure 2.

Dose response curves relating to inhalation administration of two insecticides to female mice.

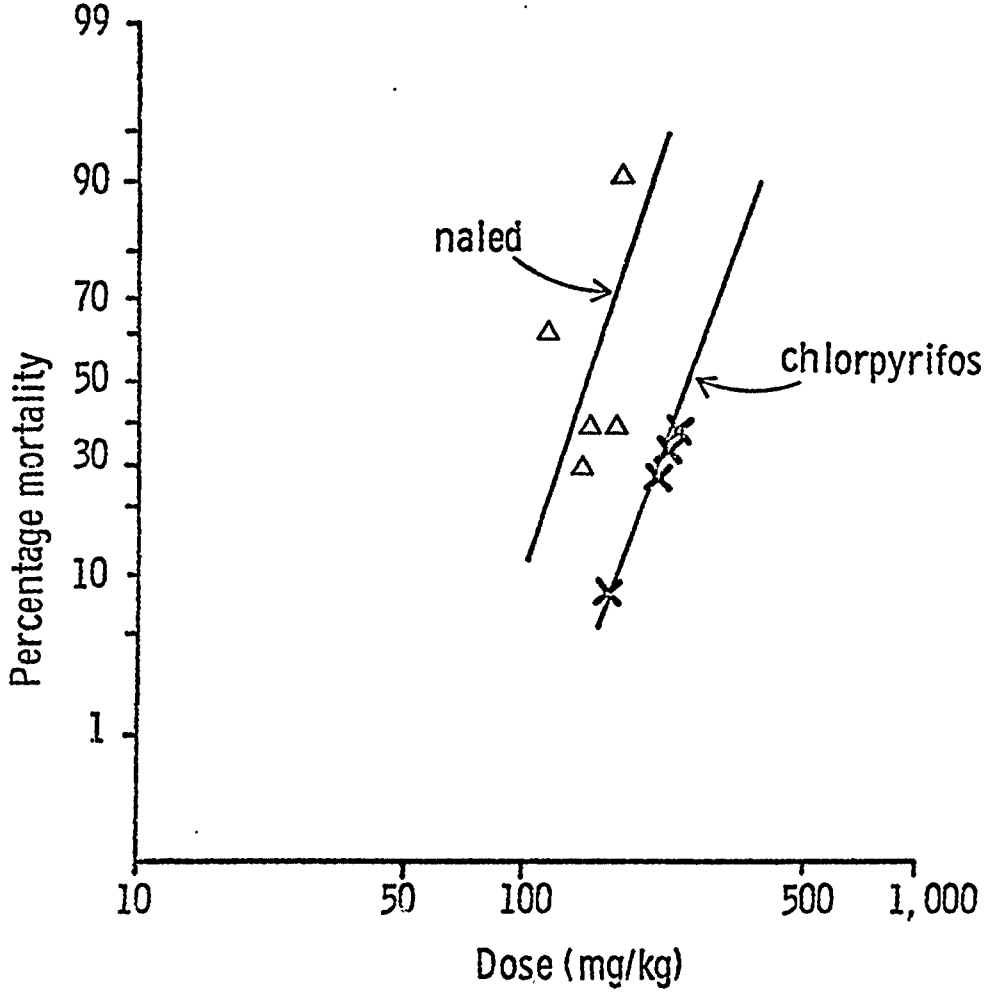


Figure 3.

Dose response curves relating to inhalation administration of three insecticides to female rats.

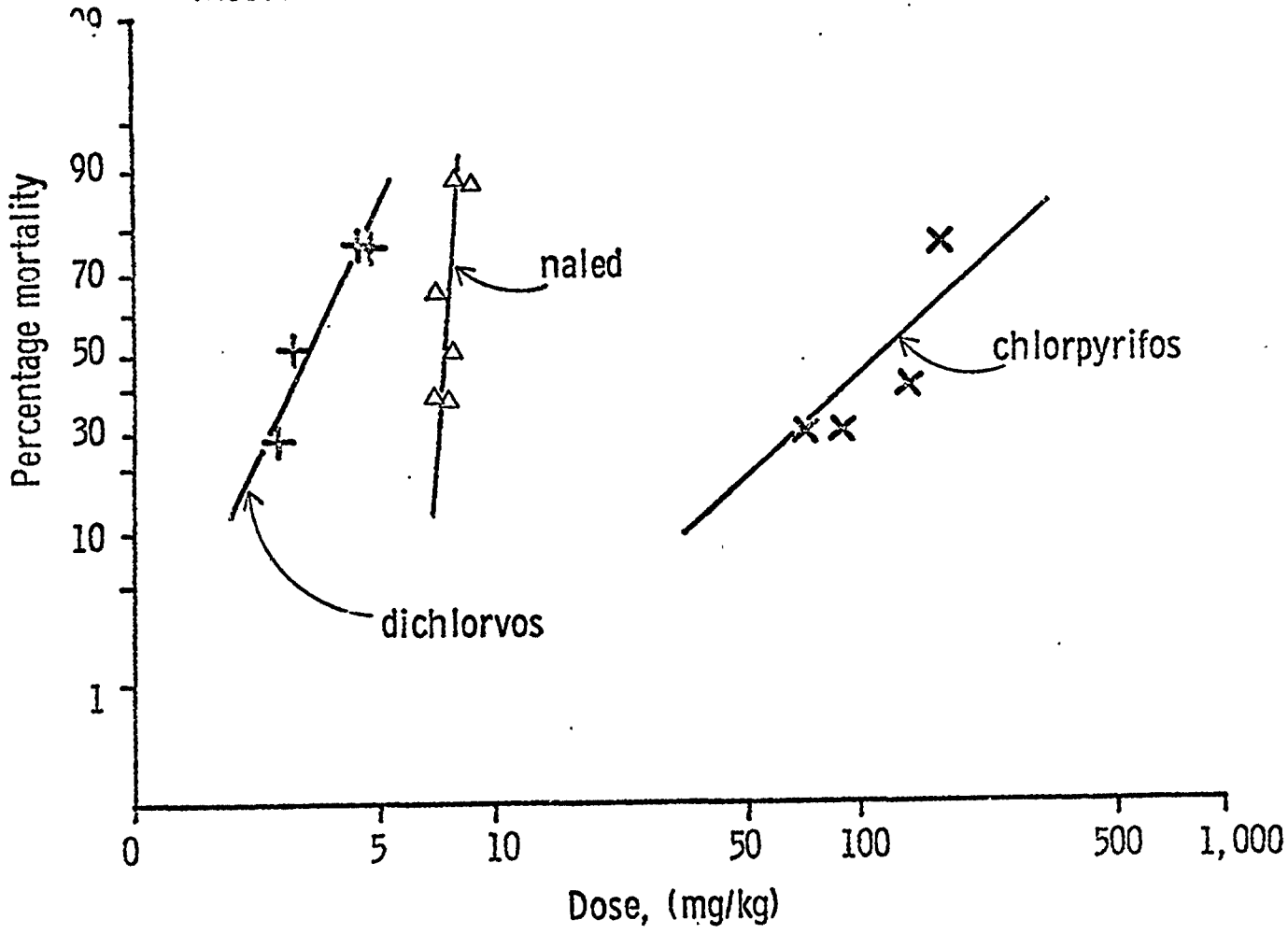


Figure 4.

Dose response curves relating to oral administration of two insecticides to female rats.

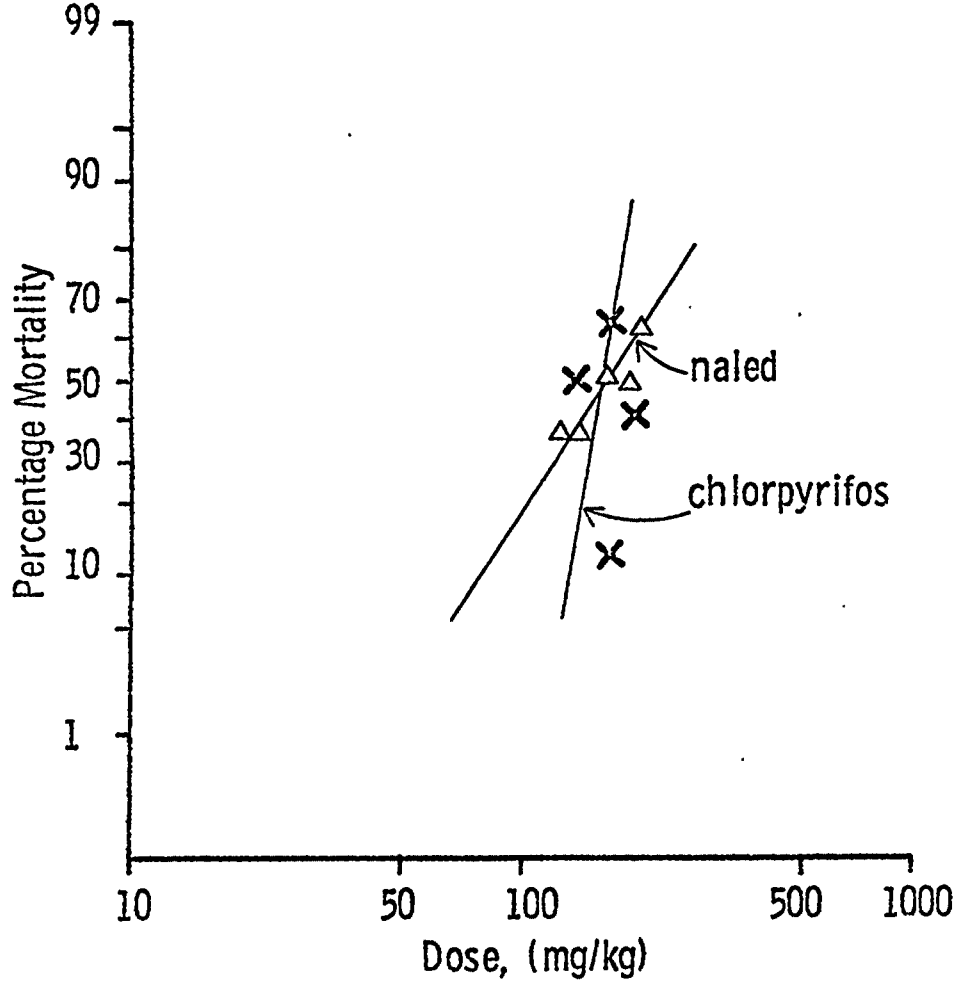


Figure 5.

TABLE 4.

INHALATION AND ORAL TOXICITIES OF FOUR PESTICIDES TO FEMALE MICE AND RATS

Species	Pesticide[a] Chemical	Toxicity (with 95% confidence intervals)			
		Inhalation toxicity[b]		Oral toxicity	Potency ratio
		LD_{50} mg/kg	LCt_{50} mg.min/m^3	LD_{50} mg/kg	Oral LD_{50} / Inhal. LD_{50}
Mouse	chlorpyrifos	257 (228-291)	2450 (2170-2760)	152 (143-162)	0.59
Mouse	malathion	>2080	19,700	1680 (1631-1730)	-
Mouse	naled	156 (141-174)	1480 (1340-1660)	222 (209-235)	1.42
Mouse	resmethrin[c]	272-665	2580-6330	1390 (1135-1703)	-
Rat	chlorpyrifos	135 (98-186)	7340 (5330-10,000)	169 (146-196)	1.25
Rat	naled[d,e]	7.7[f] (7.2-8.4)	419 (391-457)	160 (131-195)	20.8

[a] See Table 3 for formulations used in inhalation exposures: For oral toxicity determinations the technical grade pesticide chemical was formulated in peanut oil.

[b] Based upon minute volumes of 1.25 ml/min/g (mouse) or 0.65 ml/min/g (rat) (Guyton, 1947).

[c] The toxicity of xylene in which resmethrin was formulated made an accurate determination of the inhalation LD_{50} impossible (see text).

[d] Dibrom 14 concentrate (87% naled in aromatic hydrocarbons) when used alone appeared to be more toxic (see Table 5).

[e] Intraperitoneal LD_{50} 35.0 mg/kg (31.8 - 38.5).

[f] The inhalation LD_{50} of aerosols of the metabolite dichlorvos (using 10% Vapona® in soya-bean oil) was 4.03 mg/kg (3.35 - 4.53) for the rat. LCt_{50} - 219 (182-263).

TABLE 5.

EFFECT OF PARTICLE SIZE ON MORTALITY AND PLASMA CHOLINESTERASE UPON GROUPS OF EIGHT FEMALE RATS EXPOSED HEAD ONLY TO NALED AEROSOLS[a]

Dose mg/kg[b]	Duration of exposure min	Mean aerosol Concentration mg/l	MMD µm	Percentage mortality	plasma cholinesterase Percentage of pre-exposure value Mean	Standard deviation
2.36	12	1.24	2.1	12.5	69	15
3.71	15	1.56	2.1	87.5	73	6.0
5.16	21	1.55	2.1	87.5	42	8.0
10.10	65	0.98	13[c]	25	48	5.3
12.22	61	1.26	18-20[c]	25	55	11
12.35	60	1.29	18-20[c]	25	45	13

[a] Dibrom 14 concentrate; 86.2% naled in aromatic hydrocarbons

[b] Based upon 28.3% whole body retention and a breathing rate of 0.65 ml/min/g (Guyton, 1947).

[c] Approximately 7% of the particles were below 5 µm in diameter

For our own purposes, limited experiments with small particle aerosols of naled, from which a fraction of larger particles had been removed (screened aerosols), were conducted. When rats were exposed to aerosols of 10% w/v Dibrom 14 concentrate in soya-bean oil (where particles above either 2 μm or 3 μm were removed) for the same length of time that caused death of rats when no particles were removed, no rats died. When the exposure was continued for twice that time (essentially doubling the dosage) no rats died during or immediately after the exposure, but the animals became markedly agitated, and fought so intensely that it was necessary to house each of them in individual cages. Two days later all rats were dead.

Again, for our own purpose, rats were exposed to aerosols of 20% w/v of Dibrom 14 concentrate from which particles above 2 μm had been removed. In this test, a measured dosage of 7.0 mg/kg killed 62.5% of the animals -- a figure comparable to the LD50 of 7.7 mg/kg, with the 10% formulation and "unscreened" aerosols. These data provide some evidence that particles 2 - 5 μm are about as toxic, on a mass basis, as particles 2 μm and less, and that small differences in the mass distribution of particle sizes in these ranges would not be expected to significantly influence toxicity measurements.

2.2 Biochemical parameters of toxicity

2.2.1 Cholinesterase depression

Plasma cholinesterase depression in mice was most marked after inhalation of aerosols of chlorpyrifos, less marked in the case of naled, and of doubtful significance with malathion. The effect of particle size on mortality incidence and plasma cholinesterase depression when rats are exposed to naled is given in Table 5 . Figure 6 shows the relationship of cholinesterase depression to dosage (mg/kg) after mice were exposed to the three organophosphorus insecticides under study; recovery of cholinesterase from mice exposed to given doses of each of the insecticides is indicated in Figure 7.

Relationship between plasma cholinesterase depression and inhalation dose of three insecticides (female mice).

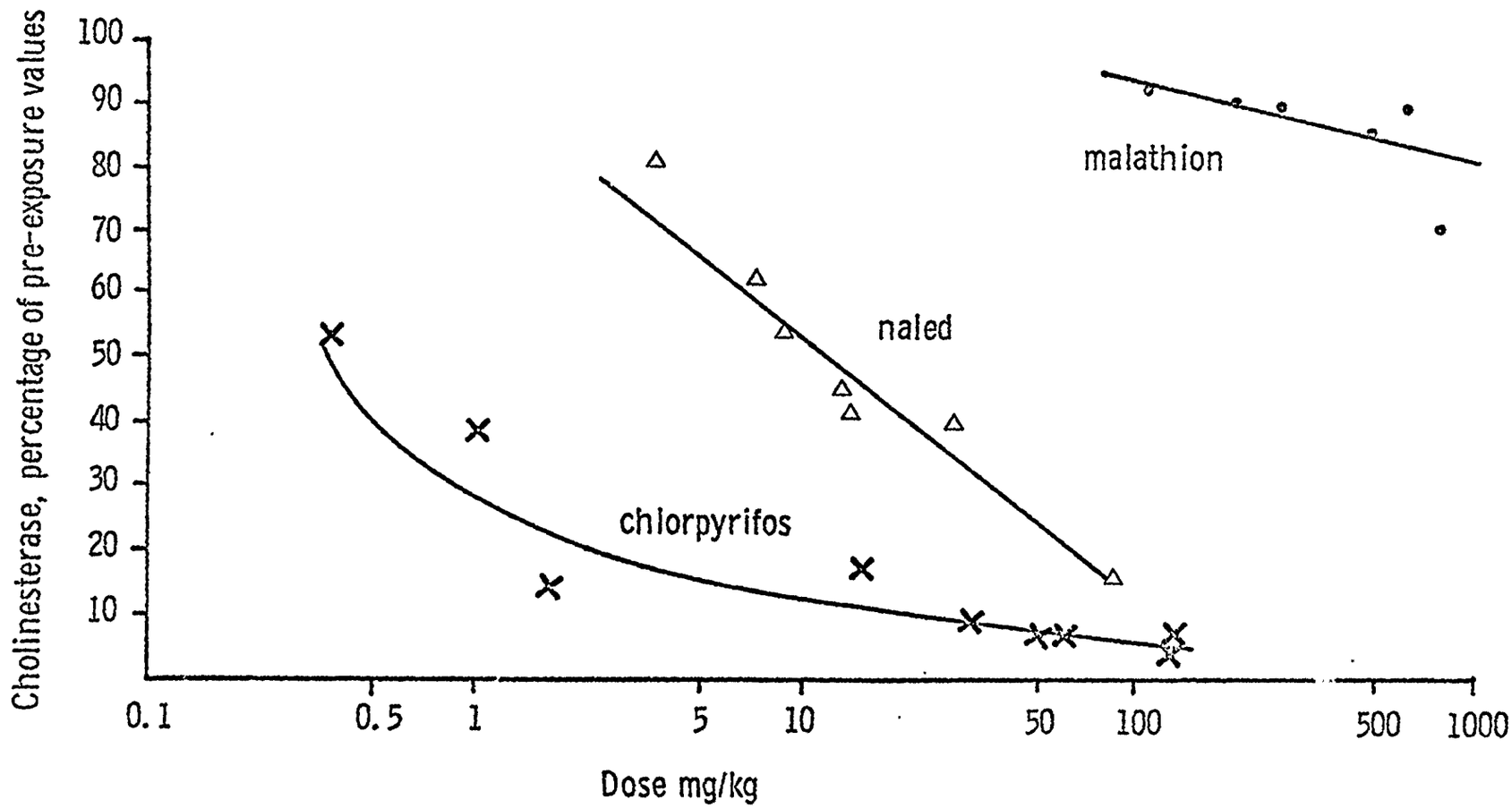


Figure 6.

Plasma cholinesterase recovery in mice following inhalation administration of a given dose of three insecticides.

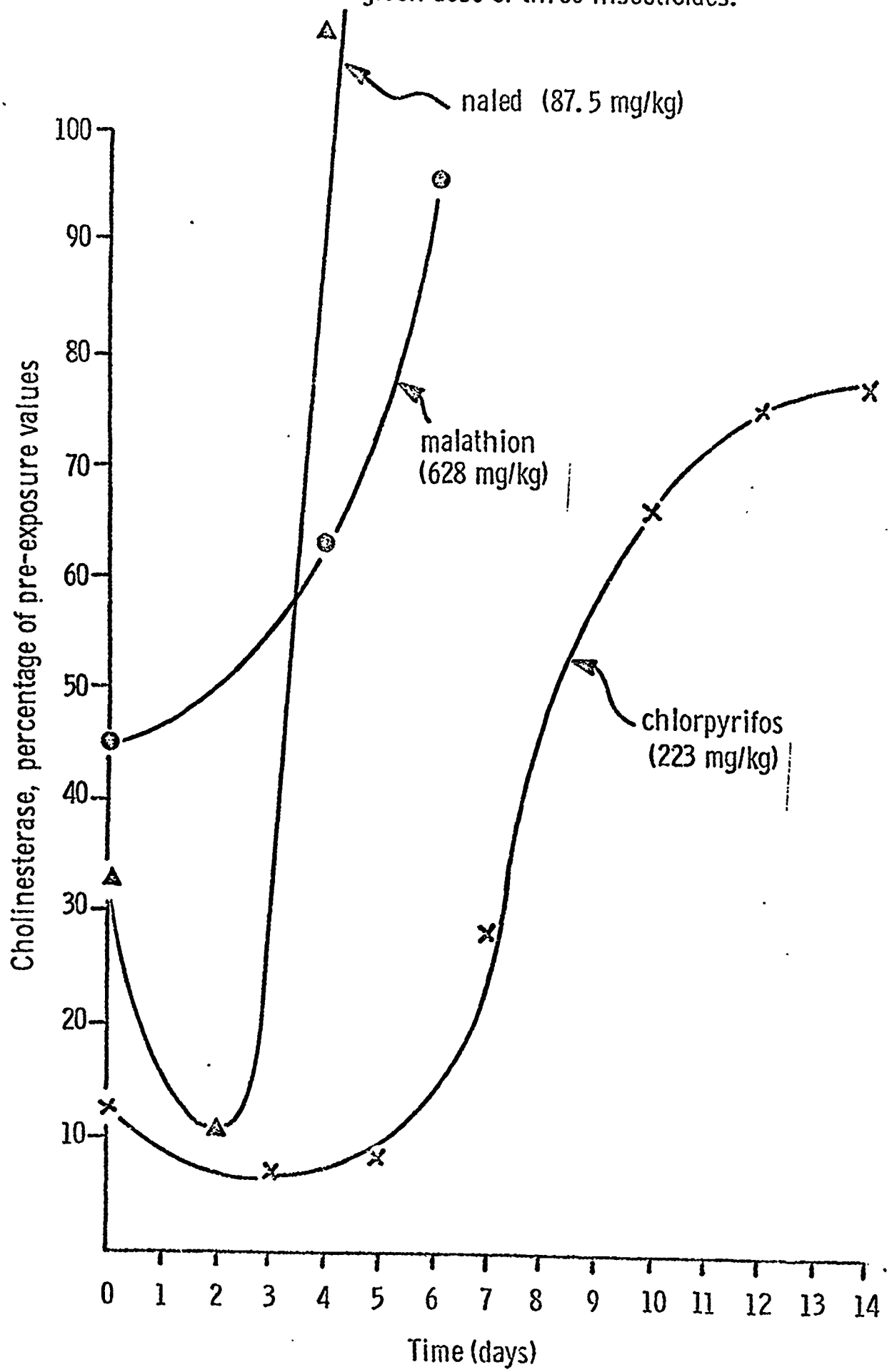


Figure 7.

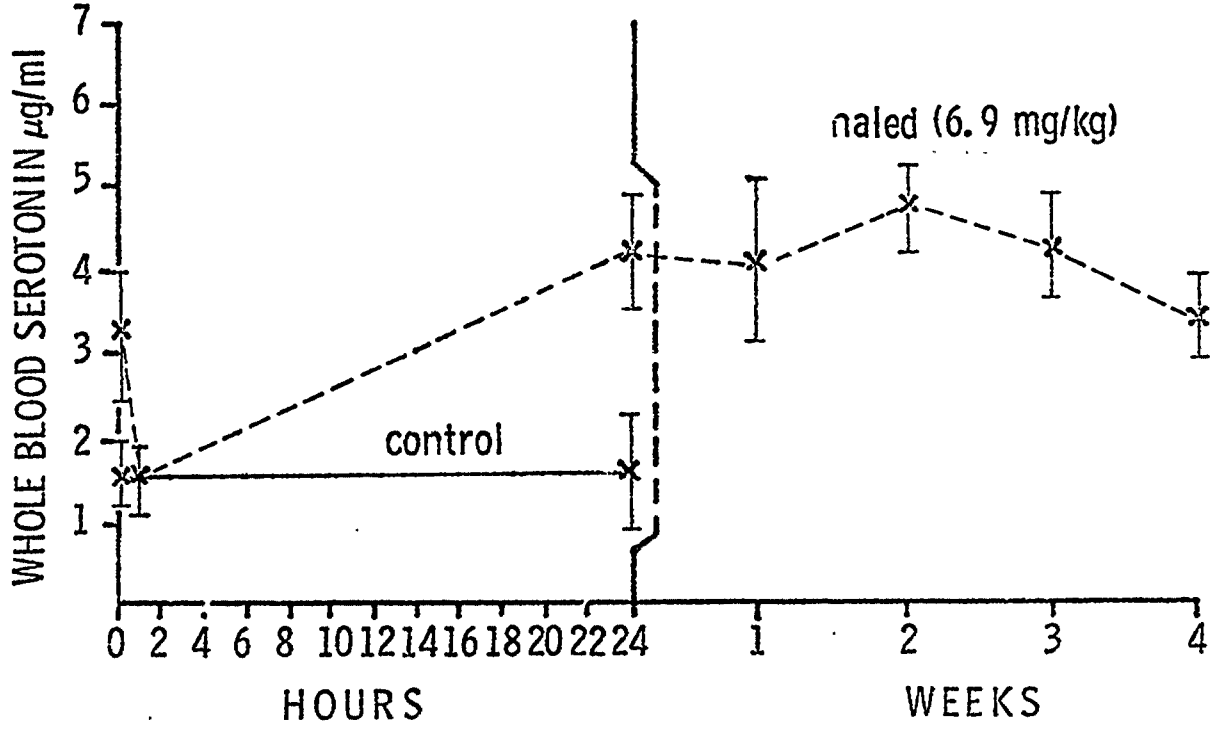
2.2.2 Whole blood serotonin levels

The effect of exposure of rats to either control or to insecticide aerosols upon whole blood serotonin levels are given in Figures 8 - 10 . These graphs also show the recovery times until return to pre-exposure values. Only chlorpyrifos and soya-bean oil alone appeared to produce no effect upon serotonin levels. With naled, a biphasic response was evident, and with malathion and resmethrin, exposure clearly elicited an increase in whole blood serotonin.

Levels of serotonin in rats exposed to resmethrin are complicated by the fact that its adjuvant, Panasol, also affected the serotonin levels. Although Panasol appears to be more toxic than resmethrin to rodents, elevation of serotonin levels was far more prolonged when resmethrin was present in the formulation. The results of this work have already been reported (Berteau and Deen, 1976). Attempts to obtain meaningful data on whole blood serotonin levels in mice exposed to insecticide aerosols were precluded because of the extreme variation in pre-exposure levels in the strain used (NAMRU).

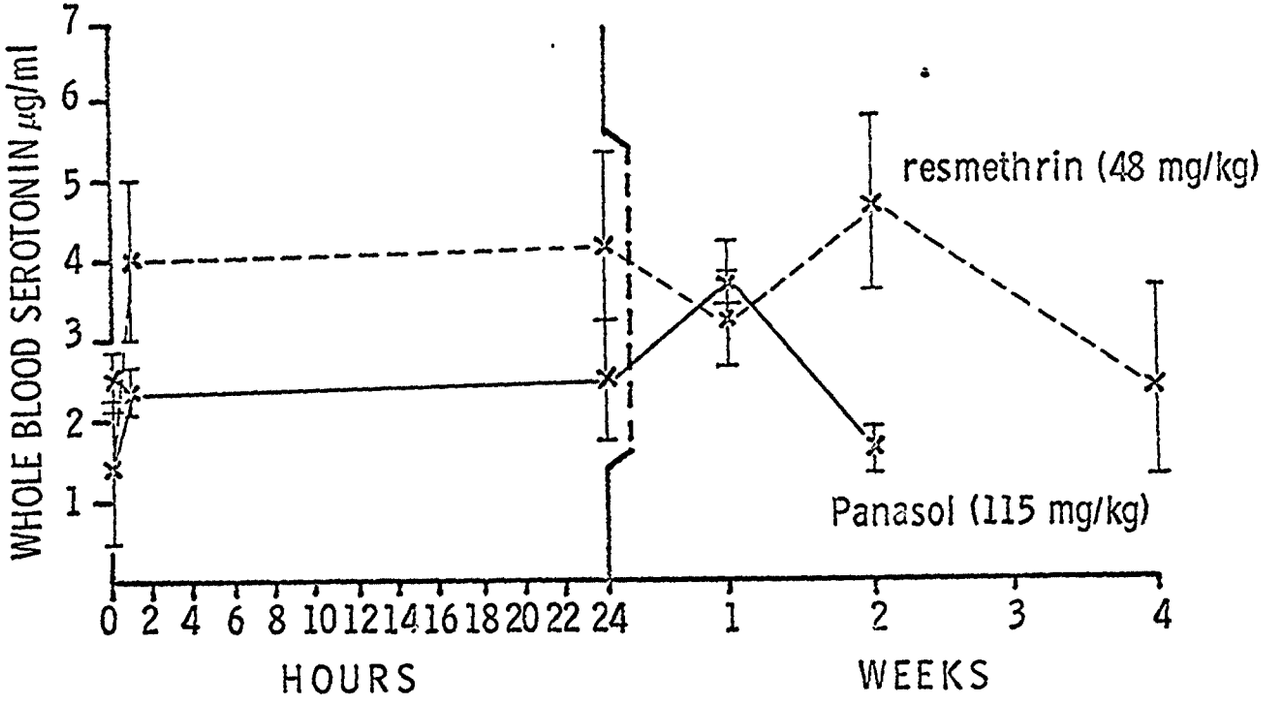
2.2.3. Whole blood glutathione levels

In a typical run with eight female mice, mean and 95% confidence intervals for whole blood glutathione (tail vein incision) was 2.72 $\pm$ 0.45 mM SH^-/l; the same values in a run with rats was more variable vis. 3.74 $\pm$ 1.43 mH SH^-/l. Inhalation exposure of mice to both naled aerosol (20% $^{w}/_{v}$ Dibrom 14 concentrate in soya-bean oil; dose 56.0 mg/kg) or to chlorpyrifos aerosol (65% $^{w}/_{v}$ chlorpyrifos in xylene; dose 325 mg/kg) produced no significant change in blood glutathione. However, after we exposed rats to naled aerosol (10% $^{w}/_{v}$ Dibrom 14 concentrate in soya-bean oil; dose 4.76 mg/kg) there was a slightly significant ($p < 0.05$; $p > 0.02$) decrease in blood glutathione. These three runs provided the only meaningful data that we generated in our studies with glutathione. In general the results were so erratic that further studies were not made. The pesticide exposure seemed to produce some obscuration of the color produced by the reagent.



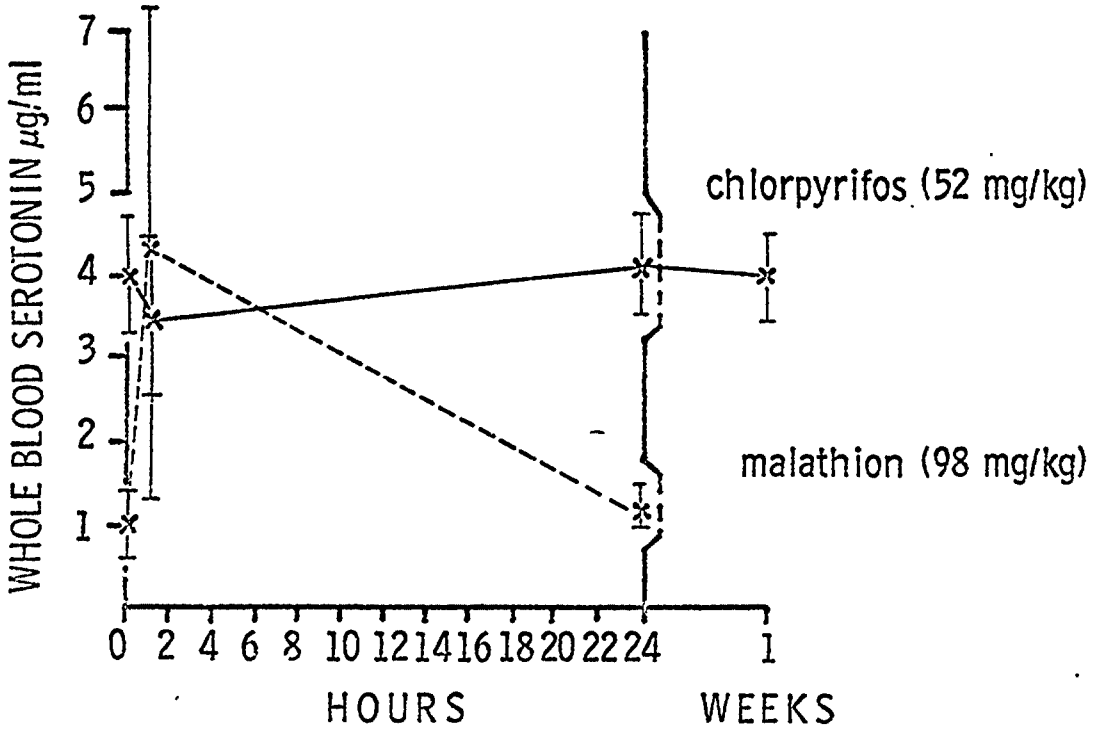
BLOOD SEROTONIN LEVELS FOLLOWING INHALATION EXPOSURE OF RATS TO A GIVEN DOSE OF NALED AND TO A SOYA BEAN OIL CONTROL.

Figure 8.



BLOOD SEROTONIN LEVELS FOLLOWING INHALATION EXPOSURE OF RATS TO GIVEN DOSES OF RESMETHRIN AEROSOL AND ITS ADJUVANT PANASOL.

Figure 9.



BLOOD SEROTONIN LEVELS FOLLOWING INHALATION EXPOSURE OF RATS TO GIVEN DOSES OF CHLORPYRIFOS AND MALATHION AEROSOLS.

Figure 10.

3. Miscellaneous determinations

3.1 Comparison of military and industrial formulations.

Based upon the gas chromatographic tracings, the military and industrial formulations of chlorpyrifos and malathion were identical. In the case of naled there appeared to be some difference in the solvent used but the concentration of active ingredient was approximately the same. The formulations of resmethrin are entirely different and the military formulation appeared to contain less of the active ingredient. We have been given to understand (Larson, personal communication) that a standard military formula exists only for malathion and this formulation has shown to be the same as that supplied by industry and is almost pure material.

3.2 Electric charge on particle deposition

A high voltage (2000 volts) applied between two aluminum disks produced a significant increase in deposition of a soya-bean aerosol on the disks; this deposition was decreased when a ^{85}krypton source was inserted in the line. The sum of the weights of aerosol deposited on all disks (less that deposited when no charge was applied) with and without the radioactive material indicated that the efficiency of dispersion of charge is about 38%. The relevance of these results to actual field conditions is doubtful because of the excessively high voltage applied; however, the effect of electrical charge is too small to be a severe limitation for defining toxicological properties expected under field conditions.

3.3 Effect of xylene on toxicity of chlorpyrifos

Table 6 gives the results of the experiment to determine if there is an effect of xylene on the toxicity of chlorpyrifos. Although there was some increase in particle size following atomization of the formulations containing xylene, the range of MMD's for all exposures where xylene concentration was increased was 1.0 - 3.0 μm. Based upon the tabulated results, as the concentration of xylene was increased (at the expense of chlorpyrifos) there was some evidence of increased toxicity contrasted with the same dose of chlorpyrifos that we achieved by increasing the exposure time. Although there was no ultimate mortality after two-hour exposure of mice to pure xylene, the animals were obviously under very considerable stress and their breathing appeared difficult. Longer exposure

TABLE 6.

THE EFFECT OF XYLENE ON THE INHALATION TOXICITY OF CHLORPYRIFOS TO MICE.

Conc. of Chlorpyrifos %	Conc. of xylene %	Conc. of Soya-bean oil %	Exposure time min.	Dose of chlorpyrifos - mg/kg	Mortality %	Mean cholin-esterase % of pre-exposure value
63	11	26	120	561	25	11.5
63	11	26	90	283	12.5	10.5
63	18.5	18.5	120	513	62.5	11.0
63	26	11	120*	530	25	11.5
63	37	0	120	743	87.5	14.6
63	37	0	90	555	62.5	10.8
63	37	0	60	426	37.5	11.5
63	37	0	50	289	0	11.1
31.5	68.5	0	97	583	12.5	12.9
16	84	0	120	418	87.5	10.7
6	94	0	60	93	0	---

*One atomizer cut out after about 100 minutes.

time to pure xylene produced mortality; 13/16 mice died after three hours exposure to atomized xylene and 4/8 rats died in a similar three-hour exposure. In such runs, the level of xylene actually encountered in the aerosol form (based upon levels found in the sampling filter) as distinct from vapor, varied considerably due to the temperature in the exposure chamber.

DISCUSSION

Effective laboratory studies of the inhalation toxicity of pesticide formulations dispersed as aerosols present certain problems not always present in inhalation studies of toxic vapors. The two most important factors are, first, that particles possess inertial properties (functions of size and density of particles) that limit the immediate transport of particles into the deeper recesses of the lungs; the breathing rate and the structure of the entire respiratory system, therefore, also limits transport. Inertial properties also limit the amount deposited in the respiratory tree -- some inhaled particles are exhaled.

Deposition for the purpose of this report is considered to be a transitory process. In most cases deposition must be measured by analysis of whole-body retention. Retention, however, is a temporal process in that material deposited in the respiratory apparatus may be absorbed or swallowed and then excreted, or it may be metabolized and lost as vapor, or the material may be transported to other tissues where it may remain for an indeterminate period. The rate of this process is both species-dependent and is distributed within the individuals of a species population. In addition, irritability of the substance may influence breathing rate, depth of deposition, etc.

A second factor is that, in practice, pesticides are formulated in a carrier vehicle; there is a concentration-per-particle effect that influences the absolute amount of pesticide deposited at a given site. Obviously, part of this effect is influenced by rate of solvent evaporation during aerosolization. It is not unreasonable to suggest that, at the deposition site, there is some innate capacity for the pesticide to be absorbed into the blood stream, and that this rate of absorption could be modified by the absolute amount of material at the site.

These problems of measurement of deposition, retention and excretion of inhaled aerosols are well known (e.g., Hatch and Gross, 1964). We have outlined them here to indicate that we are aware of the difficulties and that we have contributed additional, unsupported effort toward attempting to solve some of these problems beyond the original scope of the work.

Retention values of materials in the respiratory tree have been reported to vary from as low as 0.63% in mice, using virus suspensions in agar media (Young et al., 1974), to 50% in man, monkey and guinea pig (Hatch and Gross, 1964), or about 60% in man (Muir, 1972). In one report a retention level of 20% is given based upon a mathematical model (Anon., 1966). It has been generally accepted that larger particles tend to be deposited in the upper respiratory tract and eventually swallowed, thus entering

the esophagus and stomach. Goldberg and Leif (1950), using a particle size MMD 1.2 μm, exposed mice to an aerosol of ^{32}P-labeled Pasteurella pestis and found that of the total material retained, 30% was in the respiratory tree and 70% in the gastrointestinal tract.

We have found no reports on the retention of vegetable oil aerosols in the body; such information should be of use in determining the extent of retention of formulations of insecticides, and thus provide an indication of the hazard to the field workers. In our work, as indicated in Table 2, when mice were exposed "whole body" the majority of aerosolized material found its way into the gastrointestinal tract (mean inhaled level in the stomach, esophagus and duodenum, 70.4% out of a total of 84.1% recovered). When the animals were prevented from grooming and the major part of their bodies was protected from exposure to the aerosol, there was a slightly significant ($p = 0.02 - 0.05$) reduction in retention in the stomach; however, in this run, measurements of material in the head and duodenum were not made. Thus, it must be assumed that whereas grooming is responsible for some of the entry of the material into the gastrointestinal tract, a very significant portion of the inhaled material must have been temporarily deposited in the upper respiratory tract and then swallowed; about 10% of the total aerosol retained was actually retained in the head, presumably in the nares and nasal turbinates.

The most significant difference in retention patterns was the markedly lower retention ($p = <0.001$), the higher lung retention ($p < 0.001$), lower head retention ($p < 0.01$) and lower gastrointestinal retention ($p < 0.001$) in the rat contrasted to the mouse. This might be explained by considering the initial sites of deposition of the aerosol. At the chamber aerosol concentration encountered, ca. 4×10^6 particles /cm^3, some coagulation of particles can be expected, and coagulation is markedly increased when an aerosol is allowed to pass through an orifice (Dimmick, et al., 1975). With the mouse, the small diameter of the glottis (<0.5 mm) may act as such an orifice, creating turbulence and causing particles to coagulate and be immediately deposited in the trachea and eventually swallowed. With the rat, the diameter of the glottis is larger (2.0 mm); thus, the particles will be expected to coagulate at a lesser extent, and more will find their way into the lung of the rat. Further, we have estimated that the small diameter of the mouse trachea, ca. 1.0 mm, and the small radii of curvature before and after the glottal orifice, causes some particles of 1 μm diameter and greater to be impacted in the trachea immediately posterior to the glottis (230 cm/sec linear velocity), whereas the respiratory tree of the rat (tracheal diameter 2-3 mm) allows an increased passage of particles 1 μm or less (90 cm/sec linear velocity). Only about 16% of the mass of aerosol particles were 1μm or less but, as noted, 50% were 2.1 μm or larger. For this reason,

an approximate twofold correction could be applied to percentage of lung retention but we have not chosen to do so because the absolute values shown in Table 2 are the best values available for estimations of lung dosages under our experimental conditions.

It should be noted that, with the work of Goldberg and Leif (1950) the mean particle size was smaller (1 μm) than the MMD reported here and consequently, in our work, an increased portion of the aerosol could be expected to impact onto nasal and tracheal surfaces of the mouse, thus explaining, in part, the lower fractional lung retention and higher head retention that we encountered. The low total retention in the rats, as compared to mice, cannot be explained on these grounds. It is possible that the respiratory minute volumes under the conditions of exposure may be different than the values determined by Guyton (1947).

It is also recognized that the high aerosol concentration in the exposure chamber (4 - 5 mg/l) may have had some effect on the breathing rate leading to minute volume data different from those reported by Guyton (1947). However, in order for an animal to survive, the oxygen intake must at least be basal, which for the mouse is approximately half the resting value (basal, 1600 mm^3 of O_2/g/hr; resting 3600 mm^3 of O_2/g/hr; Dittmer and Grebe, 1958). The same fact would be true when pesticides are formulated in an oil medium; here, a possible irritant action may alter the respiratory minute volume, but again, the basal value for oxygen intake would have to be exceeded. Since the oxygen intake is proportional to the breathing rate in the same atmosphere, in computing pesticide toxicity on the basis of the retention values reported in Table 2, the maximal error in inhalation LD50 values, expressed as milligrams per kilogram of body weight, would not be greater than a factor of 2, and probably much less, because the animals did not become hypoxic.

In relating the significance of these studies to the hazard to man of exposure to toxic aerosols, it must be noted that, in the case of man, the tracheal diameter will be much larger (20 - 27 mm; Pappagianis, 1969) and thus, at the concentration examined, the percentage retention in the lung may be considerably greater.

From the information reported in Table 4, naled is approximately 21 times more toxic to the rat by the inhalation route than by the oral route. With chlorpyrifos there is only slight increase in toxicity when inhalation exposure is employed and, in view of the assumptions made in calculating inhalation toxicity values, it is doubtful if this increase is significant; mice displayed far less difference between oral and inhalation LD50 values. In fact, chlorpyrifos appears to be more toxic to mice by the oral route than the inhalation route. These facts may be explained by the

observation discussed above, that with small-particle aerosols, retention in the lung was higher in rats than in mice.

With the small-particle aerosols it is recognized that the LD50 values include some input from absorption through the skin and stomach because whole body exposure was used. However, the toxic effects of such additional absorption would be minimally significant due to the clearly more rapid effect of absorption from the lung alveoli.

Because of the high inhalation toxicity of naled (but not chlorpyrifos), factors other than rapid absorption into the bloodstream were considered. For this reason, the intraperitoneal LD50 for rats to naled was determined. Because of the extensive vascularity within the peritoneal cavity, absorption by this route involved essentially instantaneous entry into the bloodstream. Unfortunately, it was not possible to use the same solvent as was used for inhalation studies. But the solvent used, 1,2-propylene glycol, is known to have a low toxicity upon entering the bloodstream. The ip LD50 value of naled to rats of 35 mg/kg given in a foot note to Table 4 is still markedly higher than the inhalation LD50 of 7.7 mg/kg. The latter figure is based upon 28.3% retention of material inhaled; even if the retention were 100%, the inhalation LD50 would be about 27 mg/kg (still less than the ip figure). In addition, increase in the respiratory minute volume by a factor of 4-5 times would be necessary for the inhalation LD50 to approach the ip value. Such a change in ventilation was not observed.

Rapid entry of naled into the bloodstream may not be the sole reason for the high acute inhalation toxicity of this compound; the corrosive effect of naled on the lung may, in part, be a cause. The higher inhalation toxicity of naled (based on levels of active ingredient) when applied as the concentrate, rather than diluted 10% w/v in soya-bean oil, lends credence to this hypothesis. However, mere corrosive action would not explain the fact that the rats often displayed marked cholinergic symptoms and experienced acute deaths. The possibility exists that naled might be metabolized to an even more toxic material to a greater degree in the lung than in the stomach. Such a material might be dichlorvos (the debrominated derivative of naled which is known to be a transformation product of naled in the presence of compounds containing sulfhydryl groups; e.g., cysteine or glutathione). The existence of large quantities of such compounds in the lung particularly increases this possibility. Studies are being conducted to determine the in vivo and in vitro metabolism of naled when administered both into the stomach (oral) or into the lung (inhalation). The results of this work will be given in a separate report.

Plasma cholinesterase determinations in the mouse revealed that inhalation doses of chlorpyrifos far below those needed to produce

mortality produced very significant lowering of the enzyme level. This phenomenon is less marked with naled where the dose needed to kill some animals more closely paralleled that needed to produce a significant lowering of cholinesterase. Pre-exposure plasma cholinesterase levels are rapidly achieved in those animals which survived exposure to naled, but are reached more slowly after exposure to chlorpyrifos. One reason may be that chlorpyrifos is known to be retained to a limited extent in body fat (Smith et al., 1967) and thus, small but toxic amounts may be released into the blood stream for a few days following exposure.

The effect of malathion on cholinesterase depression was highly variable; in one five-hour exposure, depression was barely significant (Figure 6); however, in another two-hour exposure, the depression was 45% of the pre-exposure value. This variation may be related to the level of circulating aliesterases. These enzymes are known to detoxify malathion by hydrolyzing the ethoxycarbonyl group of the succinic acid moiety (Cook et al., 1958; DuBois et al., 1968).

The intention of this work was to determine the effect in animals of acute inhalation exposure of pesticide formulations such as might be used in ULV applications. Thus, the highest possible aerosol concentration which would not result in excessive collision of particles in the air and the shortest, reasonable exposure time (to reduce animal stress) were used. Where inhalation LD50 values have been determined, exposure times were not above three hours (Table 3). Such exposure conditions have arbitrarily been defined by us as acute conditions although it is recognized that there is no widely accepted definition of what constitutes an acute aerosol exposure.

In ULV work with ground sprayers, the MMD of the aerosols has been reported to be around 10 μm. However, such aerosols are highly heterodisperse and a significant number of particles in the 1-3 μm will be encountered. Naled is considered to be a relatively non-toxic pesticide based upon its reported oral LD50 values to rats; manufacturer's figure 430 mg/kg (Kenaga and End, 1974); Gaines (1968) reports 250 mg/kg to male rats; we found 160 mg/kg to female rats and 222 mg/kg to female mice. Based upon whole body retention after inhalation of aerosols, we found inhalation LD50 values of 156 mg/kg for mice but 7.7 mg/kg for rats. Thus, if these animal data were to be used to assess the potential hazard to humans, we would regard naled as one of the more toxic pesticides when there is appreciable inhalation exposure. It is our impression, however, that the high aerosol concentration of naled used in these studies (ca. 5 g/m^3) would seldom be encountered by field personnel for a period of time long enough for serious inhalation dosages to occur.

A major purpose of this study, namely to determine whether there is an effect of particle size upon toxicity of aerosols of insecticide

formulations, has been fulfilled only in the case of one insecticide chemical, naled. The other three insecticides were not toxic enough to enable large particle aerosols to induce mortality. Table 5 clearly indicates, however, that naled is less toxic when inhaled as particles in the 18 - 20 μm MMD range than as small (2 μm) particles, although a true inhalation LD50 for the larger particle size was not determined. Due to the lower aerosol concentration (mg/l of air) in the "large particle" chamber than in the Henderson chamber, it was necessary to make repeat runs with the small particles, but with the aerosol diluted with secondary air to make sure that the decreased toxicity of the larger particles was not some function of decreased rate of administration. Thus, the first three values listed in Table 5 refer to experiments conducted under these conditions and explain the apparent contradiction with the data reported in Table 4 . To compare the decreased toxicity of larger particles with these data is probably more valid than to compare them with the noted LD50 of concentrated aerosols. Depression of plasma cholinesterase did not parallel dose or mortality incidence. This fact lends further credence to the hypothesis that lung corrosion may play a large part in the high inhalation toxicity of naled. In fact, after exposure to aerosols of the concentrated naled (Dibrom 14 concentrate) rats that had died displayed blood stained mucus at the nose and mouth.

The purpose of the experiments where larger particles were stripped from standard aerosols was to determine whether the very small particles (< 2 μm) were more toxic than those in the 2-5 μm diameter range. The rationale was that when animals were exposed for the same time and with the same formulation as in runs used to determine LD50 values for naled, but with larger particles removed, then about the same number of animals should die if the smaller particles were the more toxic because of their enhanced penetration. No animals died under this circumstance, but when runs with "screened" aerosols were prolonged, the LD50 (mg/kg basis) was about the same as with unscreened aerosols. These data indicate, therefore, that particles in the size range of 5 - 0.8 μm are equally toxic within the limits of our measurements.

We have demonstrated, at least in the case of one pesticide, what had been predicted; that toxicity decreased when aerosol particle size increased above the 5 μm range, so the principle is established. In ULV sprays, although the MMD will be above this range, a greater number of particles will be below 5 μm than will be encountered with conventional mists. These particles will remain aloft downwind from the sprayers to a greater extent than large particles, especially in the calm air situations normally preferred during pesticide spraying. In this case, diffusion would be the major factor in decreasing aerosol concentration, and could present an increased hazard of some undetermined extent to a poorly protected worker.

CONCLUSIONS

Conclusions drawn from the studies follow.

1. The authors consider that inhalation toxicity data of particulate material are more meaningful if LD50 values are expressed as mg/kg body-weight rather than expressed from LC50 x time. A valid comparison of inhalation toxicity and toxicity from other routes of administration can be made only by use of mg/kg dosage measurements. We have shown that the determination of inhalation LD50 values (mg/kg basis) can be made with a precision of about ± 10%. The accuracy may depend on particle size, concentration of toxic substances per particle, animal species and irritant qualities that affect breathing rate.

2. The inhalation toxicity in rats of aerosols in the size range of 0.8 to 5 μm (2 μm MMD) made from 10% w/v Dibrom 14 concentrate in soya-bean oil was 7.7 mg/kg (± 0.6 mg/kg) whereas the oral toxicity of the same material was 160 mg/kg (± 30 mg/kg); thus, this material is about 21 times more toxic for rats by the inhalation route than by the oral route.

3. The inhalation toxicity in mice, with conditions as in 2, above, was 156 mg/kg (± 15 mg/kg) whereas the oral toxicity was 222 mg/kg (± 13 mg/kg); thus, this material is only slightly more toxic for mice by the inhalation route than by the oral route.

4. The inhalation toxicity in rats of aerosols in the size range 0.8 to 5 μm (2 μm MMD) made from 65% w/w of chlorpyrifos in xylene was 135 mg/kg (± 34 mg/kg) whereas the oral toxicity was 169 mg/kg (± 25 mg/kg); thus, this material is not significantly more toxic for rats by the inhalation route than by the oral route.

5. The inhalation toxicity in mice, with conditions as in 4 (above) was 257 mg/kg (± 31 mg/kg) whereas the oral toxicity was 152 mg/kg (± 15 mg/kg); thus, this material is slightly less toxic for mice by the inhalation route than by the oral route.

6. Increasing the median particle sizes of naled aerosols (Dibrom 14 concentrate) from 2 μm MMD to 13 - 20 μm MMD significantly reduced the inhalation toxicity to rats. With chlorpyrifos the concentration obtained with the larger particles was not sufficient to induce mortality

and no conclusion can be made on the impact or particle size for this pesticide. However, the inference is clear that if a pesticide is more toxic when inhaled in the form of small particles than it is by the oral route, then the larger particles of that formulation will be less toxic than the smaller ones, on a mg/kg basis.

7. When the concentration of naled in the formulation aerosolized was increased from 15% w/w to 87% w/w its apparent inhalation toxicity was increased (from LD50 7.7 $\pm$ 0.6 mg/kg to 3.1 $\pm$ 0.7 mg/kg) when comparable doses were adminstered.

8. Primary lesions in animals exposed to naled aerosols were those of pulmonary congestion and serous edema. These lesions appeared to be less severe on the basis of the dose of the insecticide chemical administered in animals exposed to the diluted formulations of naled than those exposed to the concentrate.

9. In the mouse, inhalation doses of chlorpyrifos far below those needed to induce mortality produced very significant lowering of plasma cholinesterase. This phenomenon was less marked with naled, where the dose needed to kill some animals more closely paralled that needed to produce a significant lowering of cholinesterase. Malathion produced erratic results relative to plasma cholinesterase depression.

10. Re-establishment of normal plasma cholinesterase levels in surviving mice exposed to naled areosols was rapid, but was slower in the case of chlorpyrifos.

11. Inhalation by rats of sublethal doses of aerosols of all the insecticides tested, except chlorpyrifos, caused elevation of whole blood serotonin levels. This elevation was not observed in animals after they inhaled vegetable oil aerosols. The response varied. In the case of naled, there was biphasic response; initially there was lowering of serotonin, then elevation was observed.

12. Whole body retention of a vegetable oil aerosol was markedly higher in the mouse than in the rat (84.1% versus 28.3%). However, rats retained approximately three times (9.93% versus 3.77%) the inhaled dose of the aerosol in the lung than mice did. Most of the aerosol inhaled by mice was either retained in the head or was swallowed and appeared in the stomach. This phenomenon was less marked with rats.

13. Observations on the effects of naled to pulmonary tissue after animals underwent acute exposures indicates the inadequacy of using oral toxicity data to estimate acute aerosol exposure hazards. For naled a different mode of toxicity or change to a different chemical form may be responsible for the effects observed after inhalation by animals.

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APPENDICES

SMALL PARTICLE AEROSOL EXPOSURE

1. Generation of small particle aerosols (0.8 - 5.0 μm) 3 μm MMD

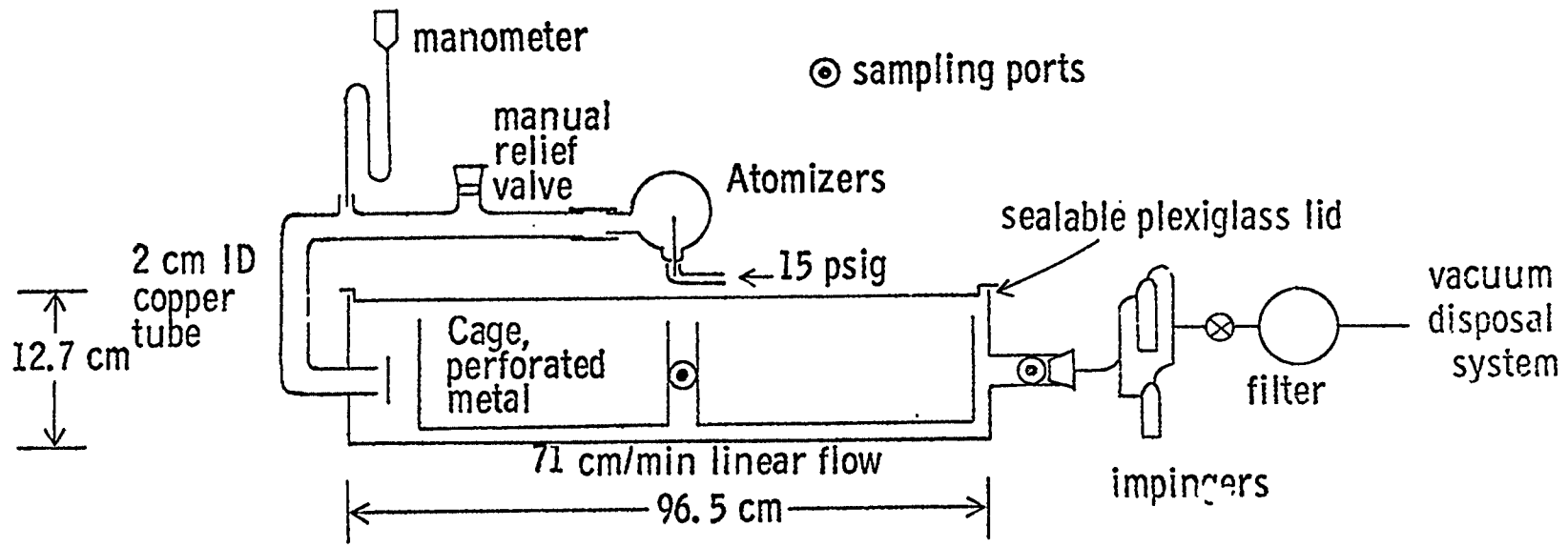
A number of atomizers are available for generating small particle aerosols. Most rely on the principle of using a jet to generate the aerosol. This aerosol is then allowed to impact upon baffles and so remove the large particles. In our work we made use of the glass Wells type atomizer (DeOme, et al. 1944) which essentially consists of a glass bulb enclosing a twin-fluid, peripheral, refluxing stainless steel jet. Details of the operation are given by Dimmick (1969). Larger particles are impacted onto the inner surface of the bulb, then returned to the reservoir of fluid to be re-atomized. With vegetable oils, we were able to obtain aerosols of MMD 1.8 to 2.2 μm and a geometric standard deviation about 2.0 μm. To obtain a relatively high rate of flow through the exposure chamber, to alleviate excessive increase in temperature of the animals, and to generate a concentration of aerosol sufficient to obtain results within reasonable exposure times, we used two Wells atomizers connected in parallel, each of which contained about 25 ml of the fluid to be aerosolized.

2. Exposure to small particle aerosols

Aerosols of diluted or undiluted pesticide formulations generated as described above were allowed to enter a modified 14.6 liter volume Henderson apparatus (Henderson, 1952; Speck and Wolochow, 1957) using an atomizer pressure of 15 lb/in^2 and maintaining the chamber under slightly negative pressure. Typical relative humidity (wet bulb) was 56%. A detailed description of this apparatus has been described (Dimmick and Hatch, 1969). Essentially, it consists of a dynamic system in which aerosol is continuously generated, allowed to enter a closed chamber in which the pressure is held slightly negative (ca. 2 cm water), moved through the chamber containing the animals at a rate of 18.6 l/min, sampled at some convenient point using Millipore* filters and the "used" aerosol disposed of by combustion (see Fig. 11).

In a typical run, the selected insecticide was placed into the atomizers which were then affixed to the input of a modified Henderson chamber (see Fig. 11). Animals were placed in cages, the cages positioned in the chamber and the transparent cover was sealed in place. The stainless steel cages were perforated on all sides, and compartmented to hold either eight mice or four rats. They were cages previously used for exposure of animals to bacteriological aerosols and had been shown not to influence the

* Millipore Corporation, Bedford, Massachussetts



Schematic of Henderson - type Chamber

Figure 11.

average flow or homogeniety of aerosols passing through the Henderson chamber. Atomizers were started simultaneously with the air exhaust system in a manner practiced to insure a slight negative pressure within the chamber. Samples were taken at appropriate times and animals were visually monitored for signs of acute distress or death. The exposure was prolonged according to the purpose of the test. At that time the atomizers were turned off and either clean air (if the animals were to be held for observation) or carbon dioxide gas (for retention studies) was admitted for five minutes, and the animals were removed.

3. Concentration and dosage measurements

To measure the concentration of pesticide chemical contained in the air, Millipore filter samples were taken periodically, usually from a side port in the Henderson chamber but, in some cases, downstream.

A 47 mm diameter, 0.8 μm pore size filter was used at a flow rate of 3.4 l/min controlled by a critical orifice attached to the vacuum line (for downstream samples the flow rate was 18 - 20 l/min.). To sample, a manual relief valve was opened, the sampling started, and the relief valve was closed while simultaneously adjusting the vacuum control valve to maintain the needed internal, negative pressure - an operation that required 3 to 4 sec. Sampling time was 1.0 or 1.5 min for side samples or 0.5 min for downstream samples. The difference in weight of the filter before and after sampling gave an approximate* value for the total amount of pesticide formulation that was collected on the filter in the given time. A more accurate value could be obtained by extracting the filter with a solvent such as hexane, making up to a known volume and chemically analyzing for the pesticide. An analytical procedure for chlorpyrifos is described elsewhere (see Appendix 7). The flow rate during sampling was measured with a rotometer. The concentration of pesticide in the aerosol is then given by

$$\text{Concentration (mg/l)} = \frac{\text{Weight of pesticide chemical on the filter (mg)}}{\text{Sample flow rate (l/min) x sample time (min)}}$$

The dose administered to the animals is then calculated assuming approximately 84% of the total inhaled in the mouse and 28% in the rat is retained, (see Appendix 6 and Results), and that the respiratory minute volume for a mouse is 1.25 ml/min/g and for a

* An assumption was made that the concentration of pesticide chemicals impacted on the filter was the same as that in the formulation atomized. This would be true only in the case of non-volatile formulations (e.g., those in soya-bean oil.).

rat, 0.65 ml/min/g (Guyton, 1947). Dose (mg/kg) = sample concentration in aerosol (mg/l) x time (min) x respiratory minute volume (ml/min/g) x $\frac{\text{Percentage lung retention}}{100}$.

After removal from the chamber the animals were normally observed for a period of two weeks to determine mortality.

4. Particle size measurement

In studies with bacterial aerosols in this laboratory, size analysis is done by sampling with Andersen samplers (Andersen, 1958) that were modified to accept plastic Petri plates. This sampler is a multi-stage sieve impactor, and has been shown to be an efficient and accurate sampler for particles in the 0.8 μm to 8 μm, aerodynamic, diameter range.

The modification was to insert pegs in each stage that would raise the plate levels to compensate for the difference between the thickness of prescribed glass plates and plastic plates. A modification made for mass analysis in this project was the addition of a thick, aluminum plate that rested on these pegs. The plate then served to raise aluminum Petri plates (trimmed to lower the top edge to an appropriate level) so that the inner, bottom surface was within the 1:5 hole-to-distance ratio prescribed by Ranz and Wong (1952) for the most effective impaction. Mass analysis of the material collected per stage was done by weight difference of the Petri plates before and after sampling. The cumulative data, as percentage of total collected, was plotted on log-probability paper to obtain a 50 percentile value, which is equal to the Mass Median Diameter (MMD). A final modification was the provision of thin (0.5 mm) disks that rested on the bottom of the Petri plates to serve as collecting surfaces. In some instances, plates were washed with aliquots of hexane and the collected material measured by chemical methods.

At the request of the sponsoring agency, the collecting and sizing capabilities of the Andersen sampler, using an aerosol of 1% aqueous ammonium fluorescein, was compared to counting and sizing particles collected on Nuclepore® filters. The amount collected on each stage of the sampler was determined by fluorometric analysis, and count and size measurements of particles on the filter were made from scanning microscopic photographs. The MMD in both instances was 1.1 μm and the percentages in each size range differed by only ± 3%. The accuracy and replicability of the sampler appears to be excellent.

* Nuclepore Corporation, Pleasanton, California

Typical insecticide aerosol data are shown in Figure 12. Curve A is the cumulative size distribution of soya-bean aerosol emerging directly from a Wells atomizer. Curve B represents a recent sample of aerosol from the exposure chamber and may be contrasted with Curve C, taken from data initially collected and reported by Berteau and Biermann (1974), who used a downstream-filter as a terminal stage for the sampler. It is evident that the distributions of particle sizes are essentially log-normal and that passing an aerosol from the atomizer to the Henderson chamber removed some of the larger particles, although the median diameters did not change significantly.

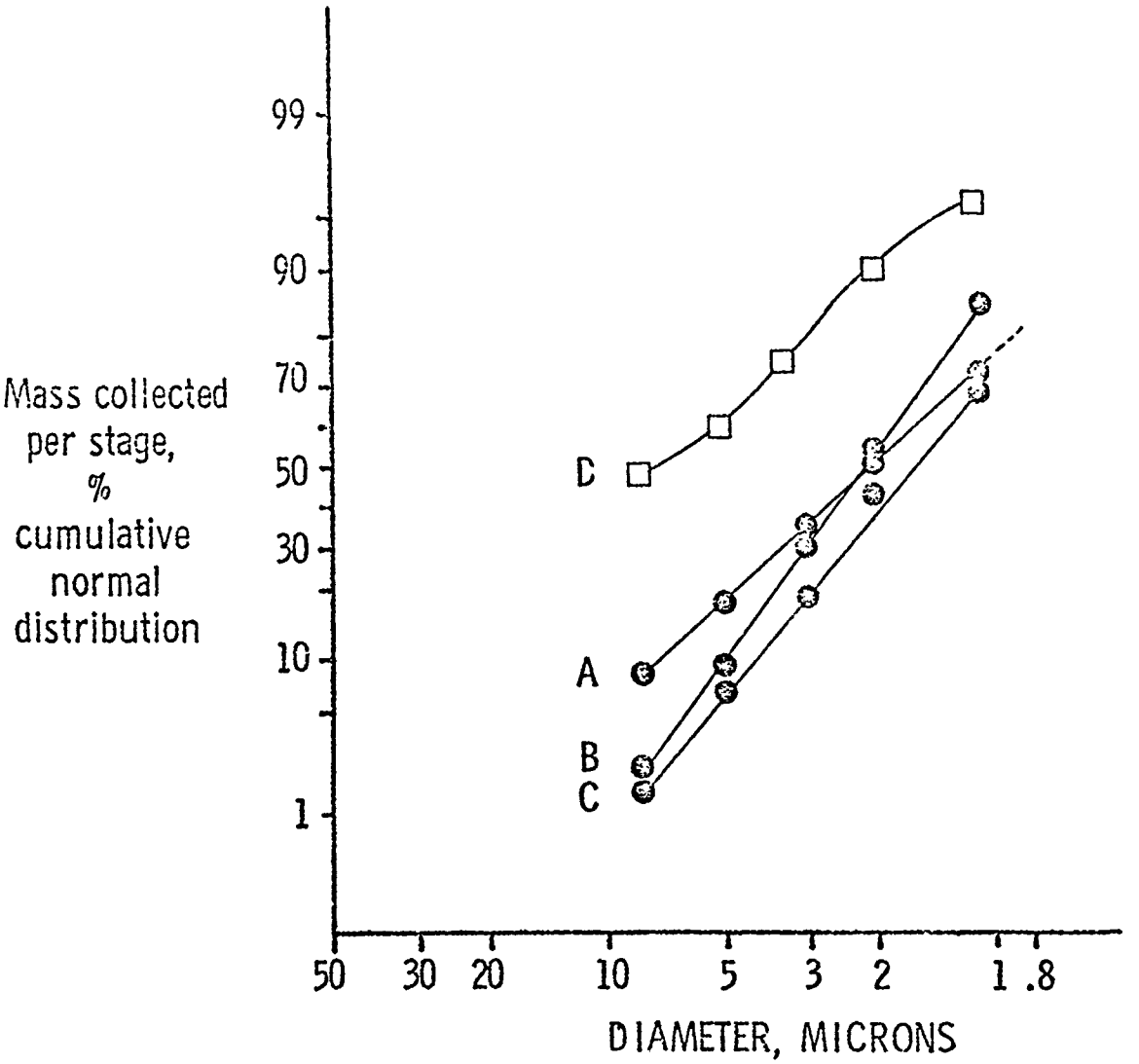


Figure 12. Size distribution of aerosols from Wells atomizer, 10% dibrom in soya-bean oil.

A. Directly from atomizer exit tube
B. In exposure chamber, 1975
C. From previous data (Berteau and Biermann, 1974)
D. Spinning disk, 25,000 rpm, chlorpyrifos

LARGE PARTICLE AEROSOL EXPOSURE

1. Generation of large particle aerosols (13 to 20 μm MMD)

To generate aerosols having particles in a defined, narrow size range, appropriate sizing methods must be available. The sizing methods, however, need an aerosol of the required size for calibration. Hence, the following is presented in more or less chronological order because at the start of the project we had neither of the methodologies and development of both progressed simultaneously.

We had anticipated that the May (1949) version of the air-driven spinning top would create particles in the desired range of 20 μm. Indeed, a slight modification of the top design yielded a stable generator, and aerosols of soya-bean oil in the 10 to 40 μm range were produced. Two problems became apparent. The first was that sizing by microscopic examination of particles allowed to settle on slides (which we had intended to use) was not successful because the large particles of the oil tended to wet the glass and to spread, so the observed circular spots of material were larger than the droplets in air. A correction factor could have been devised as is commonly done in field sampling, but not within the time limitations of the contract. More than a dozen types of surface materials were tested including Teflon® and various types of silicone oils; none was satisfactory. The data did show, however, that the predominant numbers of particles were larger than 15 μm. We then attempted to measure the size by the rate of fall of particles in a vertical glass tube; particles were observed by forward-angle, light-scatter. Data so collected indicated that the particles were in the 20-40 μm range but to have obtained a representative sample would have been too tedious and time-consuming for our purpose.

The second problem was that measurements of dispersed mass, under conditions of maximal top efficiency that produced the larger particles, showed that it would be impossible to obtain an airborne concentration sufficiently high to achieve the needed dosage level in reasonable exposure times. We decided to test a motor-driven, disk dispenser, and an ultrasonic method.

There are commercial ultrasonic dispersion units available. One is the Mist-O-Gen® generator used in inhalation therapy. A test of soya-bean oil by the manufacturer was unsuccessful. Besides being expensive, commercially available, motor-driven disk units did not fit the requirements of our chamber operation.

We obtained a Black and Decker die-grinder rated at 37,000 rpm, and also machined a stainless steel disk 10 cm in diameter that

would fit the chuck. The top of the disk was hand-ground to a velvet finish and a 2.5 mm hole 2.5 mm deep, was made in the center. The hole permitted liquid to be fed onto the disk via a small plastic tube and avoided the difficult problem of the precise positioning of a metallic feed tube as with the air-driven top.

The grinder was enclosed in a copper jacket, and a plastic shield around the disk was provided to remove satellite particles. By applying an inward airflow to the shield, smaller particles were selectively removed. Speed of the disk regulated by a variable transformer, was shown to be directly related to applied voltage. At 25,000 rpm a vibration developed that was severe enough to restrict practical usage of the disk above that speed. A sketch of the disk assembly is shown in Fig. 13.

In attempts to generate particles in the 8 to 10 μm range, the disk had to be operated at speeds higher than 25,000 rmp. Figure 12, curve D, shows that the distribution of particles from this test was not log-normal and was greater than in other aerosols. However, the noise level inside the chamber was unacceptable in the sense that the measured level above background was over 100 decibels, and would undoubtedly have caused additional stress to animals exposed to such levels. No additional attempts to create 8 μm MMD aerosols were initiated.

2. Exposure to large particle aerosols

Because of the inertial properties of large particles (greater than 10 μm) it is necessary that both sampling and animal exposure be done in such a way that particles are encouraged to always travel "downwards" and not to be rapidly diverted into bends in ducts that would remove particles. For exposure of rabbits to particles 10 μm or larger, we had designed and built an exposure chamber that conformed to these principles. The aerosol dispenser was located in the top of a chamber that had inward sloping walls leading to a restricted bottom area that would allow the exposure of heads of animals to the aerosol falling from the top compartment. This arrangement allowed the principal mass of the aerosol to drift downward so no excessive removal of large particles occurred.

The chamber was a plywood box 107 x 119 cm with an inner inverted pyramid that terminated at the bottom in a cube 23 cm per side where heads of animals could be inserted. A dispersion chamber, 38 cm per side, of Plexiglas®, and having a door that could be sealed, was centrally located at the top. Below the animal chamber another small pyramid terminated in a 4 cm ID brass tee and a valve through which air could be transported. A similar valve and tee system was located above the dispersion chamber, thus, air could be directed

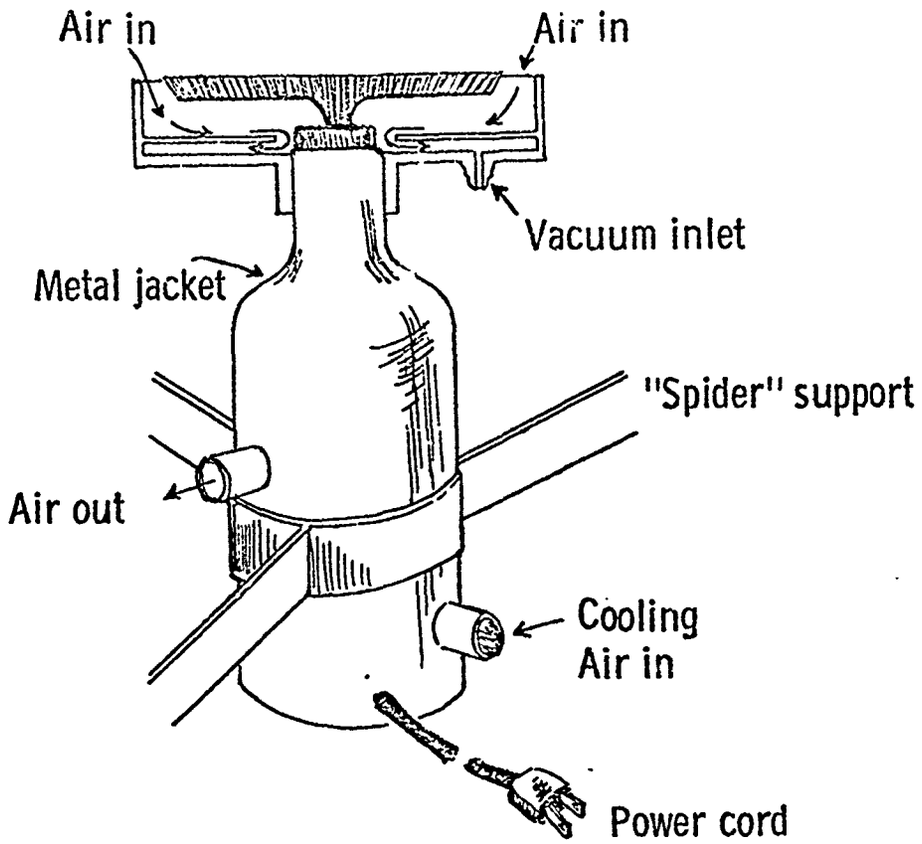


Figure 13. Conceptual sketch of spinning disk atomizer.

either upward or downward within the chamber. All inner walls, except Plexiglas, were painted with two coats of epoxy resin. The floor area outside the exposure chamber contained holes, and provision was made to transport air through the holes in an upward direction to dissipate body-heat of animals when their heads were constrained within the inner chamber. A simplified schematic illustration of the chamber is shown in Figure 14.

The chamber was modified later, at the request of the sponsoring agency, to permit exposure of rats rather than rabbits. The description of the rat-holding units is included in this section.

As shown in the Figure, the sampling port was located directly above the holding units, and the 2 cm diam. sample tube was positioned as close to the vertical as practicable, again conforming to the principle that large particles can be transported efficiently only in downward direction.

The first test of the chamber was with the air-driven top. In that test we had positioned plastic, honeycomb sheets beneath the dispenser and under the animal exposure space to encourage laminar air flow, which was in the upward direction but slow enough so particles 10 μm and larger would fall downward. Even with the limited output of the small top, it was evident that the honeycomb material collected a sufficient mass of aerosolized soya-bean oil to eventually cause dripping to occur directly on the heads of animals in the exposure space. The upper honeycomb was removed.

The first test of the disk disperser revealed another problem. The space at the top of the chamber had been designed to accommodate the air-driven top. The larger disk created an extensive air turbulence and although the "ring" of particles formed at the "stopping distance" (Sinclair, 1950) was clearly visible, particles were being swept around the dispersion chamber so violently that excess impaction occurred on tubing, wires and walls, causing additional dripping. Consequently, a drip-pan was positioned beneath the dispersion chamber, as shown in Figure 14. As a result the feed rate had to be increased and the air flow made slightly downward. The latter increased the number of smaller particles reaching the animals.

Another problem was noise generated by the disk. This was alleviated by mounting the spider on rubber pads. However, this caused the disk to absorb some of the vibrational frequency. We mounted a strobe lamp near the disk and could observe waves of fluid moving outward on the disk. Ideally, this should have been a uniform sheet. The motion undoubtedly caused additional heterogeneity in the size of the particles produced. These factors were not of sufficient magnitude to influence the exposure data more than other factors; had the LD50 values been near those of the

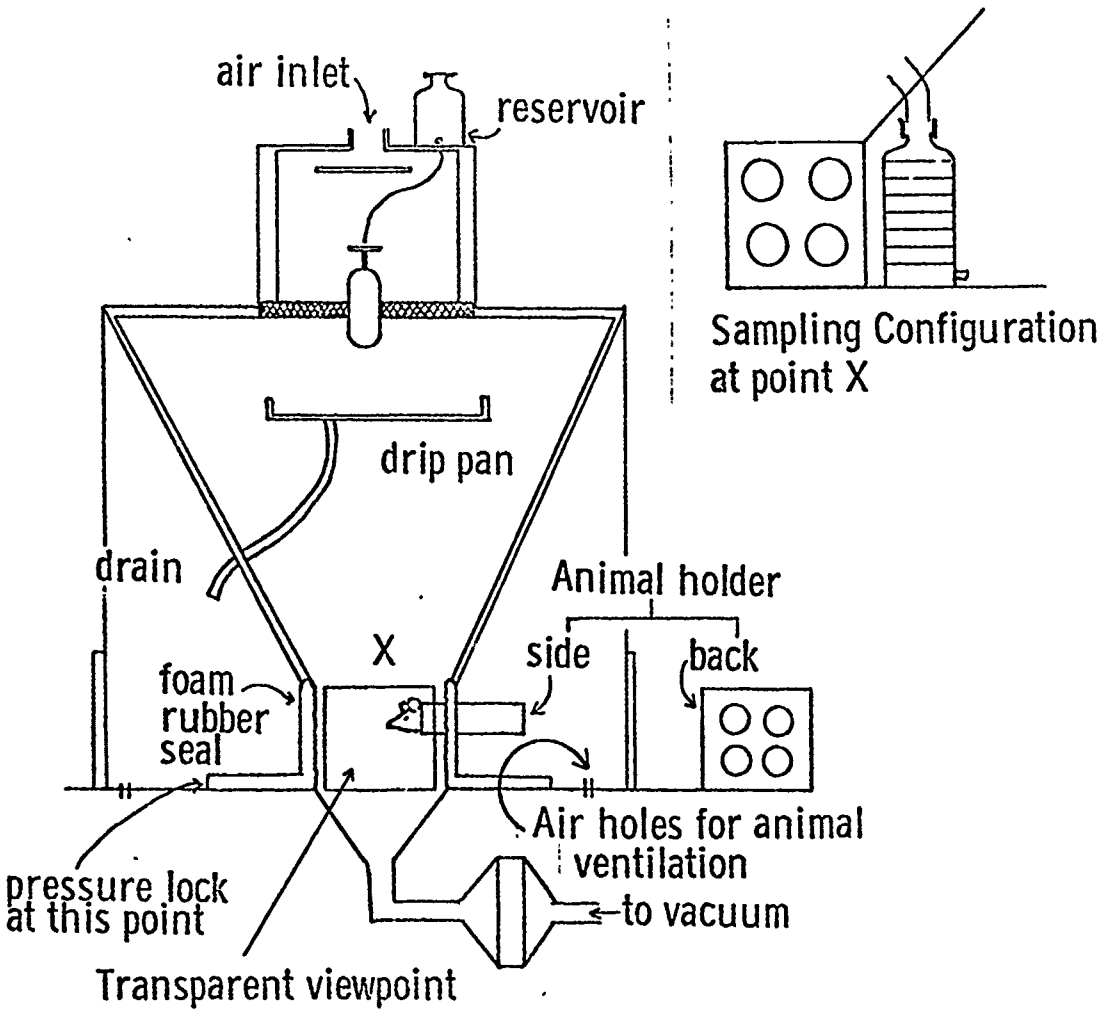


Figure 14. Conceptual sketch of modified exposure chamber.

smaller particles we would have been concerned, but since data show that the larger particles, as generated in this system, were obviously less toxic than the smaller ones, the only result is that mono-disperse, larger particles may actually be somewhat less toxic than reported here. The size distribution (Figure 15) shows, however, that only 7% of the mass of particles were 5 μm in diameter or less, hence, the difference in reported values could not be less than 7% as toxic, even if one assumed 100% retention of the mass in the smaller size range.

Animal containers for head-only exposure were similar to those described by Hoben et al. (1976). A Playtex® bottle holder and cap was used to restrain the animal with a No. 11 stopper inserted in the bottom to prevent th animal from changing position. To withdraw blood from the animal during exposure, a V-shaped slot was made in the stopper to expose the tail. The original nurser nipple was modified to act as a latex rubber gasket between the plastic cap, containing a hole through which the head protruded, and the base of the holder. The opposed longitudinal slots, originally intended for observation of the formula level, provided adequate ventilation to prevent overheating.

When used for large-particle exposures, containers were introduced into the inner, bottom portion of the chamber through a heavy rubber diaphragm, and remained in position by friction alone. This allowed variable exposure time, access to the tail for bleeding, and access to the main body of the animal for monitoring as well as allowing animals to be introduced or withdrawn as single units without affecting chamber conditions.

When these containers were used in the Henderson chamber, head-only exposure, a solid stopper was used and the body ports were closed with tape. Apparently, heat was readily dissipated to the air stream as overheating did not occur during exposures as long as three hours.

This modification has provided a convenient, semi-disposable, head-only exposure device for a unit cost of less than one dollar.

3. Concentration and dosage measurements

In operation, air was withdrawn from the bottom of the chamber at the rate of 3 l/min, or a linear, downward velocity of about 6 cm/min at the animals' heads. Because of the volume of the chamber, the aerosol concentration increased during the first 15 minutes of dispersion. Consequently the dosage was estimated from integrated analysis of the buildup. Values of aerosol concentration

*International Playtex Corporation, Dover, Delaware.

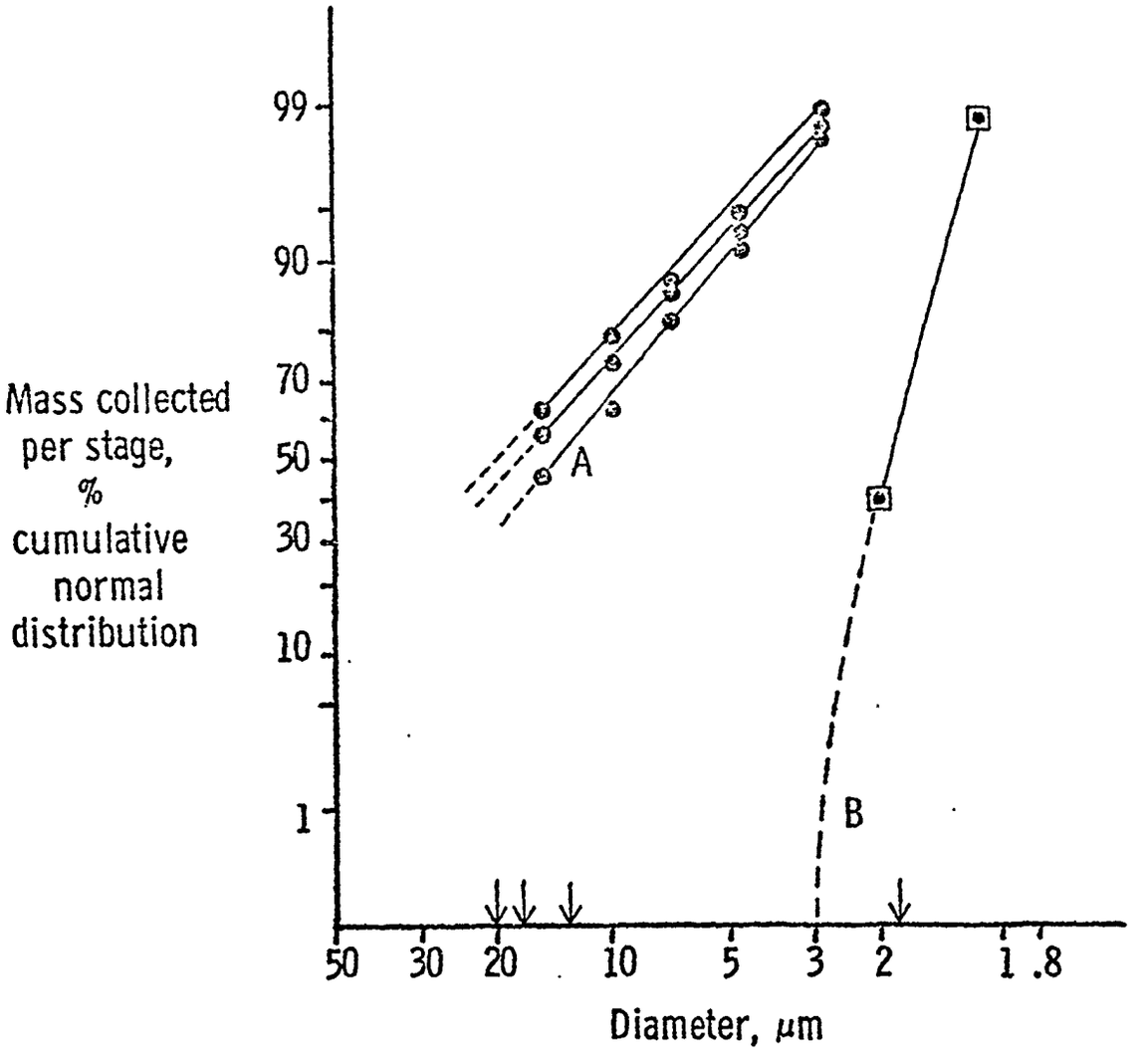


Figure 15.

●: Size distribution of aerosols from spinning disk, 87% naled, run at 16 K rpm except A (bottom set) = 23 K rpm.

B: Size distribution of aerosols from atomizer, stripped by 2-stage Andersen sampler. There were no particles larger than 2.2 μm diameter.

Vertical arrows indicate Mass Median Diameters (MMD).

for the three successful aerosols of large particles listed below were single, simultaneous samples and hence differ slightly from those reported in Table 5, which are integrated values. Pesticide was fed to the disk by a constant-head, gravity-feed device. The volume dispersed and the amount collected in the drip-pan was measured after each run.

Concentration and dosage were determined and calculated by a procedure similar to that used for small particles. During sampling the Millipore filter was attached to the chamber in the same position shown by the Andersen Sampler in Fig. 14. The sample flow rate was normally 3.5 l/min and the duration of sampling 1.0 min.

Operational Data for Large-Particle Aerosols

	Run 1	Run 2	Run 3
Volume applied to disk, ml	276	300	327
Volume collected in pan, ml	137	145	166
Dispersed, ml	159	155	161
mg/l air, by filter sampler	1.2	0.9	1.2
mg/l, by Andersen sampler	1.2	0.8	1.1
MMD, μm	13	18	20

These values show that we were able to operate the chamber in a reproducible manner and that particle "behavior" within the chamber was as we intended. During exposure of rats, a light beam from a microscope lamp was directed through the exposure space so particles were made visible by light-scatter. The particles were seen to be descending and appeared to be uniformly dispersed within the space, and remained so throughout each run.

4. Particle size measurements

While constructing and testing the disk, we also tested the use of an Andersen sampler at flow rates lower than 1 ft^3/min to determine whether this device could be used to sample particles larger than it had been designed for, and we found this procedure was possible. To calibrate this flow rate we created aerosols composed of a mixture of 1 μm, 8 μm, and 15 μm diameter, uniform polystyrene particles (Dow Chemical Co.). These aerosols were dispersed by the air-driven top, because we found only the 1 μm particles escaped from a Wells-type atomizer housing. We found that at sampling rates less than 10 l/min the distribution per stage was too broad (8 μm particles were found on stages 2, 3, 4 and a few on 5) to be useful, but at 12.5 l/min, 90% of the 8 μm particles were collected by stages 2 and 3, 98% of 15 μm particles were on stage 1 (2% on stage 2) and about 10% of the 1 μm particles were on stage 6. From these data a calibration curve for the flow

rate of 12.5 l/min (an empty impinger served as a critical orifice) was produced.

When the sampler was first used to size aerosols from the disk, we observed that a significant portion of the sample had impacted on the sieve above the first stage of the sampler and was not reaching the first collecting plate, so we constructed a pre-impaction disk that was 2 cm larger in diameter than the entrance tubing to the sampler and located 3 mm below the opening. In use, the mass collected on this disk was added to that collected on the first stage to provide a mass datum for "15 μm or larger".

Figure 15 shows data collected from runs made with the disk. The method is sensitive enough to show differences in aerosols produced at different disk speeds. Since data from all six stages were within good agreement to a log-normal distribution (which is the generally assumed variability of particles produced from liquids by application of shear forces) the pre-impaction stage proved to be an effective device. Data shown in Figure 15 was obtained during exposure of rats to those aerosols.

PLASMA CHOLINESTERASE DETERMINATIONS

The method used for determining plasma cholinesterase was essentially that of Wolfsie and Winter (1952) which is a micro-adaptation of the electrometric method of Michel (1949). A heparinized melting point capillary, 1.5 - 2.0 x 100 mm, was carefully inserted into the medial canthus of the eye of a white mouse. The orbital venus plexus was punctured and blood entered the capillary. In the case of a rat, the tail vein was incised with a scalpel and the blood released allowed to enter a capillary. When the tube was almost full, one end was sealed by insertion into clay*. The capillary was centrifuged at 12,500 rpm for three minutes when the red cells and plasma separated. The tube was then cleanly cut and 0.02 ml of plasma drawn into a Sahli pipette. The contents of this pipette were then discharged into 1.0 ml of distilled water contained in a suitable vial and the pipette was rinsed three times with the water. To the diluted plasma solution was added 1.0 ml of barbital buffer for plasma, pH = 8.00 (Michel, 1949) and the vial incubated at 25°C in a water bath for about 10 minutes. After this time 0.2 ml of 0.11 M acetyl-choline chloride solution was added and the pH read immediately to the nearest 0.01 units using a combination electrode**. The solution was then allowed to incubate at 25°C for exactly one hour to permit the enzymatic hydrolysis of the substrate. After this time the pH was read again and the cholinesterase level in the plasma expressed as ΔpH/hour. Certain correction factors tabulated by Michel (1949) to correct for nonenzymatic hydrolysis of the substrate, and corrections for variations in ΔpH/hour with pH were not normally applied since they were negligible compared to the drop in pH encountered. For a normal, healthy, non-exposed mouse the ΔpH/hour was about 2.00 units, for the rat, the figure was much lower, about 0.25 units.

After animals were exposed to aerosols of organophosphorus insecticides their blood was again withdrawn within an hour of terminating exposure and the procedure for determination of cholinesterase repeated. The ratio of the pH/hour values after and before exposure multiplied by 100 gives a figure which is indicative of cholinesterase depression and is expressed as cholinesterase percentage of pre-exposure value. A value of 80% or less was considered significant evidence of cholinergic activity. Examples of typical data generated when mice or rats were exposed to small or large particle aerosols of two insecticides are shown in Table 7 and 8.

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* Seal-ease, Clay Adams, Parsippany, N.J.

** Leeds and Northrup battery operated pH meter

TABLE 7.

EFFECT ON PLASMA CHOLINESTERASE AFTER
EXPOSURE OF MICE TO CHLORPYRIFOS AEROSOL
(PARTICLE SIZE MMD 2μm)

Formulation: 65% chlorpyrifos in xylene
Aerosol concentration: 7.87 mg/l
Exposure Time: 35 min.
Dose: 223.7 mg/kg

Mouse No	Weight	Pre-exposure pH_1	Pre-exposure pH_2	Post-exposure pH_1	Post-exposure pH_2	Δ pH/hr Pre	Δ pH/hr post	Cholinesterase % of pre-exposure value
1	33	7.83	5.47	7.90	7.52	2.36	0.38	16.1
2	31	7.88	5.62	7.88	7.54	2.26	0.34	15.0
3	29	7.83	5.68	7.86	7.58	2.20	0.28	12.7
4	33	7.85	5.85	7.76	7.44	2.00	0.32	16.0
5	31	7.86	5.67	7.84	7.52	2.19	0.32	14.6
6	31	7.88	5.61	7.88	7.54	2.27	0.34	15.0
7	30	7.84	5.86	7.87	7.59	1.98	0.28	14.1
8	28	7.89	5.82	7.73	7.49	2.07	0.24	11.6
9	30	7.87	5.70	7.81	7.55	2.17	0.26	12.0
10	32	7.90	5.73	7.87	7.57	2.17	0.30	13.8
11	32	7.86	5.75	7.88	7.73	2.11	0.15	7.1
12	30	7.90	5.89	7.85	7.60	2.01	0.25	12.4
13	32	7.90	5.63	7.86	7.60	2.27	0.26	11.5
14	33	7.90	5.74	7.78	7.63	2.16	0.15	6.9
15	33	7.93	5.79	7.71	7.51	2.14	0.20	9.3
16	32	7.87	6.03	7.63	7.48	1.84	0.15	8.2
Mean								12.3
S.D.								3.0

TABLE 8.

EFFECT ON PLASMA CHOLINESTERASE
AFTER EXPOSURE OF RATS TO NALED
AEROSOL (PARTICLE SIZE, MMD 18 - 20μm)

Formulation: Dibrom 14 concentrate
Aerosol concentration: 1.3 mg/l
Exposure time: 60 min.
Dose: 12.4 mg/kg
Mortality: 2/8

Rat No.	Weight g.	Pre-exposure pH_1	Pre-exposure pH_2	Post-exposure pH_1	Post-exposure pH_2	Δ pH/hr pre	Δ pH/hr post	Cholinesterase % of pre-exposure value
1	259	7.90	7.49	7.80	7.55	0.41	0.25	60.9
2	269	7.92	7.02	7.87	7.43	0.90	0.44	49.0
3	298	7.93	7.23	7.91	7.65	0.70	0.26	37.2
4	295	7.91	6.97	7.93	7.69	0.94	0.24	25.5
5	280	7.89	7.10	7.98	7.69	0.79	0.24	30.4
6	292	7.93	7.36	7.89	7.64	0.57	0.25	43.9
7	284	7.94	7.41	7.89	7.59	0.53	0.30	56.6
8	290	7.93	7.51	7.91	7.68	0.42	0.23	54.8
Mean								44.8
S. D.								12.8

WHOLE BLOOD SEROTONIN DETERMINATION

Female white mice of the NAMRU strain weighing 30 - 40 g, or female white Sprague-Dawley rats* weighing 250 - 350 g were utilized for these studies. Blood from the mice was obtained from the orbital venus plexus by insertion of a capillary tube into the medial canthus of the eye. With rats, blood was obtained by incision of the tail. Exactly 20 µl was drawn into a Sahli pipette. The method for determining serotonin was essentially the modification by Krueger et al. (1963) of the method of Undenfriend et al. (1955). To 25 ml of a 1% solution of ethylenediaminetetraacetic acid was dissolved 0.75 g of L-ascorbic acid. (This solution must be used within 2 hours of its preparation). For an experiment involving 8 animals, 1 ml of this solution was placed in each of 8 stoppered centrifuge tubes, the 20 µl contents of the Sahli pipette discharged into the solution and the pipettes rinsed 3 times with the solution. To each tube was added 0.5 ml of borate buffer (pH 9.5) which had previously been saturated with n-butanol and sodium chloride. The solution was shaken briefly, 1.5 g of sodium chloride added followed by 3.0 ml of n-butanol saturated with water. The tubes were stoppered and mechanically shaken vigorously for 10 minutes. Eight other tubes were prepared containing 4.0 ml of n-heptane saturated with water and 1.5 ml of 0.1 N hydrochloric acid. After shaking was complete we centrifuged the tubes for five minutes, removed 2 ml of the butanol top layer and added that to the heptane-hydrochloric acid mixture in the other tubes. These tubes were then shaken vigorously for five minutes, the heptane layer aspirated and the serotonin level in the aqueous lower layer read with a spectrophotofluorometer** at 295 nm excitation and 335 nm fluorescence. The fluorescence range was scanned from 300-350 nm. A standard curve was prepared each day using the same procedure except that in place of the blood samples we added 20, 40, 60, 80, and 100 µl of a solution containing 2.5 µg/ml serotonin (equivalent to 0.05, 0.1, 0.15, 0.2 and 0.25 µg of serotonin). A blank sample containing no serotonin was also prepared. A stock solution containing 10 µg/ml of serotonin was prepared weekly by dissolving 11.5 mg of serotonin creatinine sulfate complex*** in 500 ml of 0.1 N hydrochloric acid. The n-butanol and n-heptane were "chromatoquality" grade.

The animals (normally 8 in number) were exposed in the Henderson chamber, the aerosol being generated from two Wells atomizers in parallel. Concentration and dose were obtained from sampling on Millipore filters as described in Appendix 1, Section 3.

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* Charles River Laboratories
** Fluorispec Model SF-1, Baird Atomic Inc., Cambridge, Mass.
***Sigma Chemical Company, St. Louis, Mo.

WHOLE BLOOD GLUTATHIONE DETERMINATION

The method used was adapted from the methods of Ellman (1959). Blood was withdrawn from mice or rats by tail incision; 2 µl was added to 0.9 ml of distilled water and 1.0 ml of phosphate or Hepes buffer, pH 8.0, was added. In each of two Beckman 1 cm cuvettes was placed 3 ml of this solution. Two-hundredths ml (0.02 ml) of a solution containing 39.6 mg of 5,5' dithiobis-2-nitrobenzoic acid* in 10 ml of phosphate buffer (pH 7.0) was added to the blood sample. The absorbance from the red color developed was measured zeroing the other cuvette with the untreated portion. Results may be expressed as mM of SH^-/l. of blood from the formula:

$$c = 36.8\ A$$

where c = concentration
and A = absorbance

The constant (36.8) was verified by plotting a standard curve from a known solution of glutathione.

The lack of consistency of this method is described in the Discussion section. Attempts to use the modification of Beutler et al. (1963) were without success.

*Aldrich Chemical Company, Milwaukee, Wisconsin

APPENDIX VI

DETERMINATION OF WHOLE BODY RETENTION OF AEROSOLS

To determine an inhalation dose of an aerosol in an animal in terms of mg/kg body-weight, it is necessary to know the respiratory minute volume of the animal under the conditions of exposure and also the fractional retention of the material breathed. The former figure was not determined in this study, but the generally accepted values of Guyton (1947) applicable to resting conditions was used. In the case of retention, however the values reported (see Discussion) vary so greatly with the materials used that we decided to determine values using the major adjuvant of our formulations, namely, soya-bean oil. To do this procedure, use was made of a radioactively labeled tracer. Initially 1-^{14}C-dodecanol* was used but due to the possibility that this compound might be metabolized and the label lost as ^{14}C-carbon dioxide a more inert tracer was substituted. Most of the recent retention studies have now been made with 1-^{14}C-heptadecane.* To a 50 ml volumetric flask was added 0.5 mCi of 1-^{14}C heptadecane in about 0.15 ml of benzene. About 5 ml of unlabeled heptadecane was added and the solution made up to volume with soya-bean oil.

Sixteen mice or eight rats were placed in the compartmented cages of the exposure chamber and were exposed as described above. At least 25 ml of soya-bean oil containing the 1-^{14}C-heptadecane was placed in each of two atomizers, the aerosol generated, and animals were exposed for about 20 minutes. During that time two or three filter samples were taken on 0.8 μm, 47 mm diameter Millipore filters. The aerosol leaving the chamber was passed through two impingers which removed almost all particles and the residual was passed through a filter before the air was transmitted to the vacuum system. After the exposure was complete, atomizers were turned off and, simultaneously, carbon dioxide gas was allowed to enter the chamber from a cylinder. By this means the animals were sacrificed as rapidly as possible after exposure to the radioactive aerosol. With mice, death resulted in less than one minute; with rats, slightly longer. Carbon dioxide was allowed to pass for five minutes and then the chamber was air-washed for a further five minutes. The animals were removed and lungs, trachea, stomach, esophagus and duodenum were dissected out. Heads were also removed. In some cases a portion of liver and a blood sample were taken. Lungs of mice were divided into three to five portions, the stomach into two and the trachea and esophagus were used whole. Each portion of tissue was placed in a plastic disposable scintillation vial and digested by standing for 24 hours with Unisol† tissue solubilizer. With rats known fractions of lungs and stomach (approximately 100 mg) were digested whole with 1 ml of tissue solubilizer. Fur

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*Purchased from ICN Isotope and Nuclear Division, Irvine, California.

†Isolab Inc., Akron, Ohio.

was removed from the heads and they were digested 1-4 days in tissue solubilizer (10 ml for mice or 50 ml for rats) and after digestion the material was made up to ten times its volume with Unisol complement. Filter samples were digested with 2 ml of methanol. To the tissue material in the vials (except the heads), 0.5 ml of methanol and 10 ml of Unisol complement (scintillation fluid) was added for each ml in the vial. For the heads 10 ml portions were placed in the vials. Three drops of 30% hydrogen peroxide were added to the finished cocktail to remove color and diminish quenching. Materials in the vials were counted in a Packard Tri Carb scintillation counter set to maximum gain for ^{14}C. In one run a Beckman LS 250 scintillation counter was used. To determine quenching for the material in each vial, 1 ml of a standard solution containing known counts was added to each vial containing tissue, then if

$$q = \frac{\text{Actual counts}}{\text{Counts with quenching}}$$

we could determine the true (unquenched) counts for each vial from the equation:

(Counts in vial after addition of standard) x q =

Standard + (Counts in vial before addition of standard) x q.

This last term in this equation is the desired result. The radioactive count of the aerosol was calculated from the equation:

$$\text{Aerosol concentration (cpm/ l)} = \frac{\text{Mean counts/min on the filter}}{\text{Sample flowrate (l/min) x sample time (min)}}.$$

Then, for an animal mass m,

$$\text{Total cpm breathed} = \frac{\text{Aerosol concentration x m x breathing rate (ml/min/g) x time of exposure (min)}}{1000}$$

The actual corrected cpm in each vial was summarized for each organ; then:

$$\text{Percentage retention in organ} = \frac{\text{cpm in organ (corrected) x 100}}{\text{cpm breathed}}$$

The mean background radioactivity was determined for each run and this amount was subtracted from each vial reading. For liver samples, the whole organ was weighed and a weighed portion only (ca. 100 mg) digested. With blood, the quenching was too great because of the heme present, so only plasma was counted.

At the end of the exposure, the Henderson chamber was closed, the aerosol regenerated and the size distribution determined using an Andersen sampler. Plates were extracted 3 times with hexane, the extracts made up to 10 ml and counted. The proportion of counts on each stage was then utilized to determine the size distribution as described above.

Tables 9 and 10 give the respective levels retained in organs or tissues in a given population of mice or rats.

TABLE 9.

PERCENTAGE RETENTION IN VARIOUS ORGANS OF TOTAL CALCULATED INHALED AEROSOL[a] IN FEMALE MICE

Mouse no.[b]	Percentage retention in tissue[c]						
	Head	Lung	Trachea	Esophagus	Stomach	Duodenum	Total
1	2.47	3.99	1.34	0.44	90.85	1.33	100.42
2	7.28	4.26	2.85	0.21	53.62	14.20	82.42
3	10.50	3.90	0.50	1.10	102.96	3.98	122.94
4	12.84	4.18	0.06	1.02	37.01	12.10	67.21
5	12.80	3.07	0.91	0.62	75.79	2.62	95.81
6	7.21	3.01	0.33	0.90	36.14	14.20	61.79
7[d]	-	-	-	-	-	-	-
8	10.30	3.98	0.08	28.33	15.21	0.24	58.13
Mean[e]	9.06	3.77	0.87	4.66	58.80	6.95	84.10
95% conf. int.	±3.43	±0.46	±0.91	±9.67	±29.68	±5.80	±21.93

[a] Soya-bean oil containing 1-^{14}C-heptadecane; aerosol of MMD 2.1μm
[b] Mean weight of mice, 30.5g
[c] Based on respiratory minute volume 1.25 ml/min/g (Guyton 1967)
[d] Incomplete and anomolous results led to rejection of data on this animal
[e] Mean values and 95% confidence intervals following head only exposure were: lung, 4.6 ± 0.86; trachea, 1.2 ± 0.91; stomach, 39.8 ± 13.1; esophagus. 1.4 ± 0.75; total (excluding head and duodenum), 46.9 ± 13.0. (See Discussion).

TABLE 10.

PERCENTAGE RETENTION IN VARIOUS ORGANS OF TOTAL CALCULATED INHALED AEROSOL[a] IN FEMALE RATS

Rat no.[b]	Percentage retention in tissue[c]						
	Head	Lung	Trachea	Esophagus	Stomach	Duodenum	Total
1	5.53	8.24	0.09	0.29	14.44	1.59	30.18
2	2.05	7.15	0.18	0.04	11.10	0.87	21.39
3	3.91	10.91	0.33	1.34	16.24	0.90	33.63
4	3.27	10.42	0.10	1.15	33.04	3.15	51.13
5	2.41	8.96	0.09	0.06	4.45	2.12	18.09
6	1.62	7.02	0.10	0.01	11.70	0.95	21.40
7	2.63	9.71	0.17	0.11	6.20	0.36	19.18
8	3.43	17.02	0.19	0.11	5.57	5.12	31.44
Mean	3.10	9.93	0.16	0.39	12.84	1.88	28.31
95% conf. int.	1.03	2.67	0.07	0.47	7.70	1.32	9.19

[a] Soya-bean oil containing 1-^{14}C-heptadecane; aerosol of MMD 2.1µm
[b] Mean weight of rats 364 ± 37 g
[c] Based on respiratory minute volume 0.65 ml/min/g (Guyton 1947)

MISCELLANEOUS DETERMINATIONS

1. Analysis for chlorpyrifos

This method was essentially the same as that described by Dow (1972) except that hexane was used as a solvent instead of methanol because the latter material destroyed Millipore filters during extraction. A standard curve was prepared by accurately weighing 125 mg of an analytical sample of chlorpyrifos*, dissolving in hexane and making up to 250 ml with analytical grade hexane. Dilutions of 0.2 ml, 0.5 ml, 1.0 and 2.0 ml were made up to 10 ml with hexane. These solutions then comprised concentrations of 1.0, 2.5, 5.0 and 10.0 mg/100 ml. The absorbance was read at 289 nm against a hexane blank using a Beckman DB spectrophotometer. Values were plotted on linear graph paper to give the standard curve. Beer's Law was well observed over the range of 1 to 5 mg/100 ml.

2. Comparison of formulations

Samples of pesticide formulations for use in the toxicological studies discussed were normally obtained direct from the manufacturer. However, military formulations were obtained from Aberdeen Proving Ground, Maryland, and it was desired to know if the formulations were the same. For this purpose, 0.1 µl of the formulation was injected into a Hewlett Packard F and M Scientific 700 laboratory gas chromatograph having a Teflon column of dimensions 50 x 0.32 cm., containing 15% SE 54 on 100/120 gas chrom Q. The injector temperature was 140°C, detector 180°C and the column oven temperature was programmed from 100 - 200°C. The tracings were compared for each sample injected.

3. Partial dispersion of aerosol charge

In order to try to disperse any electrical charge that may have been present on particles of oil aerosols generated in Wells atomizers, we used a krypton^{85} source. To determine the effectiveness of this procedure, an apparatus was prepared such that an aerosol was allowed to pass between two pairs of electrically charged aluminum disk plates. The plates were weighed and then the aerosol was allowed to pass for 6 hours with no charge applied. A constant vacuum was applied to draw the aerosol through. Initially no charge was applied. The plates were weighed at the end of the experiment. The experiment was then repeated with the charge of 2000 volts applied with and without a krypton^{85} source in the line. The distance between the mouth of the atomizer and the plates was kept the

* The analytical sample of chlorpyrifos was kindly supplied by the Dow Chemical Company.

same for each experiment. The efficiency of the radioactive source in dispersing the charge was determined by the reduction in weight of the plates when the source was present.

4. Effect of xylene on the toxicity of chlorpyrifos

One of the standard formulations of chlorpyrifos is a 65% $^{w}/_{v}$ solution of the active ingredient in xylene. In early inhalation exposure runs there was some indication that chlorpyrifos may become more toxic when its formulation was further diluted with xylene. To examine this possibility, technical grade unformulated chlorpyrifos was used. The compound was then formulated by us, using various concentrations of xylene and a nontoxic vegetable oil adjuvant (soya-bean oil). These formulations were atomized and eight mice exposed in the Henderson chamber, as previously described, to the small particle aerosols generated. Cholinesterase measurements were made and 14-day mortality recorded. Andersen samples before and after atomizing for the given exposure time were taken for most runs.

5. Particle screening

The LD50 values reported for naled using Wells atomizers indicated that about 1.0% of the particles were 4 μm or larger, although the MMD was 2 μm. To determine if the toxicity of a 10% $^{w}/_{v}$ solution of Dibrom 14 concentrate in soya-bean oil would increase if these particles were removed, four runs were conducted in the Henderson chamber. In these runs, before allowing the aerosol to enter the chamber, a number 3 or a number 4 stage of the Andersen sampler was placed in the aerosol line to screen particles 3 μm and larger, or 2 μm and larger, respectively. Sampling for aerosol size and density, and determination of toxicity, was as described previously.

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